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TITLE: Role for a Steroid Sulfontransferase (SULT2B) in the Intratumoral Androgen Metabolism and in Prostate Cancer

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14. ABSTRACT

We are investigating the role of a steroid-metabolizing sulfotransferase (SULT2B) in intra-prostate androgen metabolism and in metastatic castration-resistant prostate cancer (mCRPC) stimulated by androgen-activated androgen receptor. SULT2B-mediated sulfation of dehydroepiandrosterone (DHEA), the precursor steroid for the active androgen 5α-dihydrotestosterone, should reduce intra-prostate DHEA levels, thereby inhibiting androgen production and stimulation of mCRPC by androgen receptor. We find that SULT2B is completely absent in distant metastases from mCRPC patients, suggesting a role for SULT2B in mCRPC. Causal association of SULT2B with mCRPC is indicated by our preliminary data, which show that xenograft tumors from SULT2B-knocked down CRPC cells grew faster than tumors from SULT2B-intact CRPC cells. In another notable result, CYP17A1 and HSD3β1, two key enzymes in the androgen biosynthesis pathway, were robustly induced in CRPC cells by androgen and vitamin D, respectively, and these inductions were prevented by co-treatment of cells with androgen and vitamin D. Concurrent presence of androgen also prevented vitamin D-mediated induction of CYP24A1, which converts 1α,25-dihydroxyD3 (active vitamin D) to an inactive metabolite. These results are potentially significant, suggesting that a combined vitamin D and androgen regimen may offer a novel therapy against mCRPC. Preclinical studies in mice are under way to test this possibility.

15. SUBJECT TERMS

mCRPC; SULT2B; DHEA, 5α-DHT; tissue microarray; immunohistochemistry; tumor xenograft; androgen metabolism; androgen receptor; vitamin D receptor; gene regulation

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Introduction: The project aims to decipher the role of the steroid sulfotransferase SULT2B1b (hereon SULT2B) in metastatic castration-resistant prostate cancer (mCRPC). SULT2B, a prostate-expressed sulfotransferase, has strong substrate preference for dehydroepiandrosterone (DHEA), which is the steroid precursor for testosterone and more importantly, 5α-dihydrotestosterone (DHT), the active prostatic androgen. SULT2B-mediated catalytic conversion of DHEA to a sulfated metabolite should reduce the supply of DHEA for de novo androgen biosynthesis. Thus, SULT2B activity may play a role in the growth of mCRPC, which harbors active androgen receptor (AR) signaling fueled by intratumoral DHT, despite the castrate level serum androgen. A role for SULT2B in prostate cancer was strengthened by our result (reported in 2013) that SULT2B expression is reduced significantly in hormone-naïve primary prostate cancer. Our new data shows that SULT2B is completely lost in most mCRPC tissues from distant metastases (procured as part of a rapid autopsy program).

Using CRPC cells and CRPC xenograft tumors, in ongoing studies we are evaluating the impact of the loss of SULT2B on CRPC tumor growth *in vivo* and CRPC cell proliferation *in vitro*. Since SULT2B expression in prostate cancer cells is induced by the ligand-activated vitamin D receptor (VDR) and liver X receptor (LXRα) (our results), and LXRα-mediated gene regulation is antagonized by AR (based on literature reports), we are also exploring how AR ⇔ VDR and LXRα ⇔ AR interactions influence intratumoral androgen metabolism and the growth of mCRPC.

Keywords:
mCRPC; SULT2B; DHEA, 5α-DHT; tissue microarray; immunohistochemistry; tumor xenograft; androgen metabolism; androgen receptor; vitamin D receptor; liver X receptorα; gene regulation

Accomplishments: At year-1 of the DOD support, our study provides new evidence for a role of SULT2B in the regulation of CRPC growth, and suggests a potentially new approach for therapeutic intervention of CRPC, as explained below.

I. *Complete loss of SULT2B expression in mCRPC isolated from distant metastases*:

IHC staining of SULT2B in tissue microarray (TMA) of 160 mCRPC samples, recovered from distant metastases after rapid autopsy, shows complete loss of SULT2B expression in most patient samples. TMA slides were obtained under MTA with UW (Seattle) as part of PI's collaboration with Dr. Elahe Mostaghel, MD, PhD, who is a well-known clinical prostate cancer researcher and member of the Fred Hutchison Cancer Center. This result contrasts our results on hormone naïve primary prostate cancer where SULT2B expression in cancer tissue is significantly reduced but not completely absent compared with SULT2B expression in matched non-cancer tissue. Thus it appears, SULT2B expression is progressively lost in parallel to prostate cancer progression. **Figure 1** is a representative result showing complete loss of SULT2B expression in mCRPC.

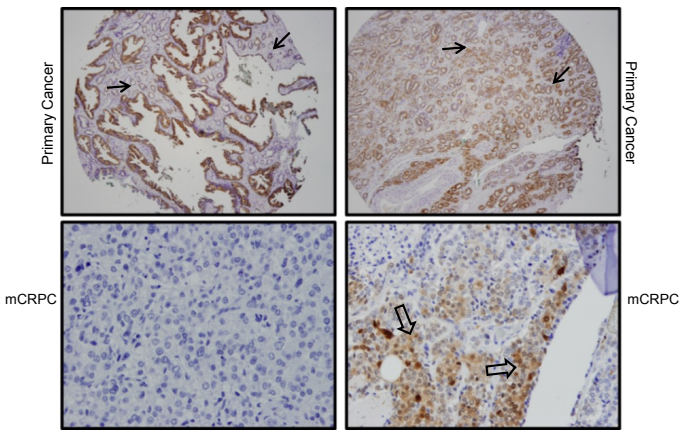


Fig 1. SULT2B-immunostained prostate cancer specimens. **Upper panel**: Primary prostate cancer (hormone naïve) from two patients. **Lower panel:** mCRPC, (distant metastases) from two patients. Upper panel in the left shows both non-malignant acini, which stained strongly for SULT2B, and cancerous areas (solid arrows), which show markedly reduced SULT2B levels. Upper panel, tissue core at the right shows only the malignant region of primary cancer (arrows), which stained weakly for SULT2B. Photomicrographs taken at 4X. **Lower panel**: The left panel shows complete absence of SULT2B; right panel shows some residual SULT2B (open arrows). Photomicrographs at 20X.

Work in section I is in compliance with Task 1b, Task 2b, Task 2c of approved SOW.

Experiments are ongoing to explore a causal role of SULT2B loss in mCRPC.

II. ***Correlation of SULT2B loss with increased proliferation rate of DHEA-stimulated CRPC cells and increased tumor growth rates of CRPC xenografts in immune-deficient nude mice***.

(IIa) Generation of CRPC cells with stable knockdown of SULT2B:

CRPC clones with stable SULT2B knockdown were prepared by infecting two different AR-positive CRPC cells (C4-2 and C4-2B) with lentivirus-expressing shRNAs that are complementary to SULT2B mRNAs at the 3' end. Cells were infected separately with each lentivirus sample expressing one of the 5 shRNAs specific to 5 different regions of SULT2B mRNAs. Control clones arose from cells infected with the lentivirus that expresses non-targeting shRNAs. Several clones from C4-2 and C4-2B cells showed marked reduction of SULT2B levels, as revealed from western blot assay (**Fig. 2**). Two SULT2B-silenced clones from each cell line and corresponding non-targeting clones showing high SULT2B levels were selected for analysis.

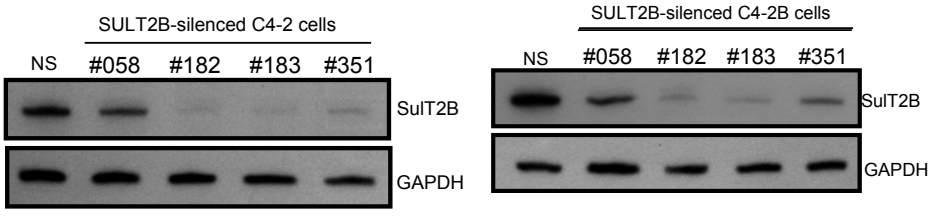


Fig. 2. SULT2B levels, determined from western blot experiments in C4-2 and C4-2B CRPC cells with or without SULT2B knockdown. Individual clone numbers are indicated. NS= non-specific targeting shRNAs.

IIb) Proliferation of CRPC cells with or without SULT2B knockdown:

Cells in RPMI-1640 media containing 5% charcoal-stripped serum were treated with DHEA at 5 μM and trypan blue excluded cells were counted (automated cell counter, Invitrogen) at day- 1, 2 and 5. **Result**: SULT2B-silenced cells proliferated more rapidly compared to control, SULT2B-intact cells.

Data interpretation: when cells are in charcoal-stripped serum, exogenously supplied DHEA is the sole precursor source for DHT production. Thus, DHEA supports DHT-stimulated CRPC cell proliferation. Upon SULT2B knockdown, a greater amount of DHEA is available for DHT synthesis, hence causing a higher proliferation rate for SULT2B-silenced cells compared to SULT2B-intact cells. These preliminary data will be repeated with exogenously added DHEA at different concentrations (0.1, 0.5, 1, 2 & 5 μM) in order to find the optimal DHEA concentration which provides a maximum difference in the proliferation rate between SULT2B-knocked down versus SULT2B-intact cells. Assays will be run in triplicates with 3 different clones in order to get statistically significant results.

IIc) Xenograft tumor growth rate with SULT2B-silenced versus SULT2B-intact CRPC cells.

SULT2B-silenced and SULT2B-intact C4-2B and C4-2 cells, mixed with matrigel at 1:1 (V/V) were injected subcutaneously in athymic nude mice (male), and tumor volumes were measured beginning at ~150-200 mm^3 tumor size. Volume = ½(length x $width^2$); length = maximum longitudinal diameter; width = maximum transverse diameter. Tumors were produced with several clones for each cell line.

Results: In several mice, tumors with SULT2B-silenced cells grew at a faster rate. However, we did not get statistically significant results since such differences in tumor growth rates were not detected for a number of other mice. This problem was exacerbated by the fact that tumor take rate was low (~60-70%), and for those mice which developed tumors, tumors grew steadily only for approximately half of them. For the other half, tumors either did not increase in size, or tumors shrank, rather than growing steadily, for a number of mice. We will repeat this experiment with NOD-SCID mice, which are more

severely immune deficient than athymic nude mice and thus, a higher tumor take rate is expected. In this case, we can analyze more number of mice, and generate statistically significant results.

IId) Steroid profiles of SULT2B-silenced and SULT2B-intact cells

As a first attempt, levels of testosterone, DHT, 17α-hydroxypregnenolone, DHEA for hormone naïve primary prostate cancer (5 samples) and matched non-cancer prostate tissue (5 samples) were assayed using Core Service at UW-Seattle. Results show higher testosterone and 17α-hydroxypregnenolone levels in tumor tissue compared to matched non-malignant tissue. However, more samples should be analyzed in order to reach statistical significance ($p \leq 0.05$) for these differences.

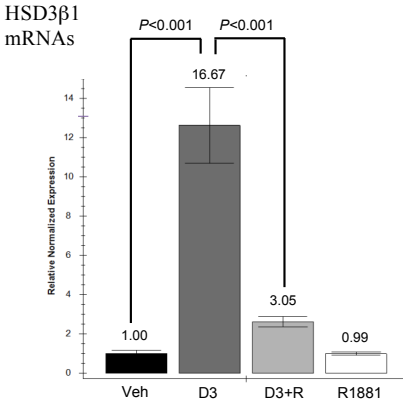
Cell pellets from SULT2B-intact and SULT2B-silenced CRPC cells will be sent out to our collaborator (Dr. Elahe Mostaghel) for assay of the levels of steroids in the androgen biosynthesis pathway (pregnenolone, 17α-hydroxypregnenolone, DHEA, DHEA-sulfate, androstenedione, testosterone, DHT) using Core Service at UW-Seattle.

Work conducted for IIa, IIb, IIc, IId is in compliance with Task 1a, Task 1c, Task 4a, Task 4c

III. ***Novel interplay of androgen- and 1α,25 D_3-mediated regulation of enzymes driving androgen biosynthesis and vitamin D metabolism in CRPC cells***

Inhibition of CRPC by 1α,25-D_3 (hormonally active vitamin D) has been widely documented in experimental models. In human trials, however, vitamin D therapy was ineffective against prostate cancer, which is in part due to hypercalcemia that develops at a high clinical dose of 1α,25-D_3. In new data, we show that two key enzymes in the androgen biosynthesis pathway, namely CYP17A1 and HSD3β-1, as well as CYP24A1, the enzyme that metabolizes 1α,25-dihydroxy D_3 to the inactive metabolite (1,24,25 trihydroxy D_3) are regulated by 1α,25-D_3 (vitamin D) and androgen in a manner that may be exploited for therapeutic opportunity (explained below).

Results: HSD3β1, the enzyme that converts DHEA to androstenedione, was induced ~16-fold by 1α,25-D_3 in C4-2 and C4-2B CRPC cells, while R1881 (a synthetic androgen) induced CYP17A1 by ~6-fold. CYP17A1 is the rate-limiting enzyme in the androgen biosynthesis pathway, catalyzing, through its 17α-hydroxylase activity, the conversion of pregnenolone to 17α-hydroxypregnenolone, which is further metabolized to DHEA, catalyzed by the 17, 20 lyase activity of CYP17A1. R1881 alone did not induce HSD3β1, and 1α,25-D_3 did not alter the basal CYP17A1 level. In contrast, in combined treatment, 1α,25-D_3 and R1881 together prevented HSD3β1 induction by vitamin D, and CYP17 A1 induction by R1881 in CRPC cells. These results (**Fig. 3A, 3B**) suggest that a therapeutic regimen of combined vitamin D and androgen can potentially suppress two key enzymes in the androgen biosynthesis pathway, thus reducing intratumoral androgen production in prostate cancer.



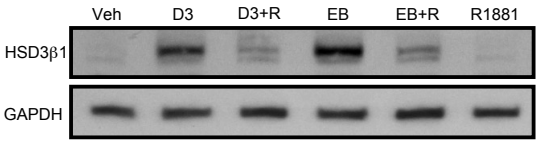


Fig. 3A. Left: HSD3β1 mRNAs assayed by quantitative RT-PCR. Data are normalized to β actin mRNAs. D3= 1,25vitamin D3; R= R1881. Right: Western blot assay for HSD3β1 protein. EB= EB1089, a synthetic vitamin D.

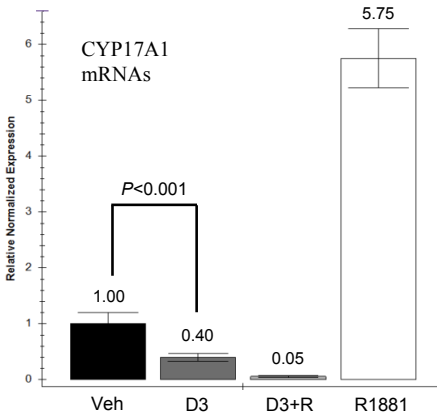


Fig. 3B. CYP17A1 mRNA levels assayed by quantitative RT-PCR, using β actin mRNAs as the normalizing control.

Our result reveals a second potential benefit of the combined vitamin D and androgen regimen, since CYP24A1 induction by vitamin D was abrogated in cells treated concurrently with R1881 and 1α,25-D_3 (**Fig. 3C**). CYP24A1 mediates metabolism of 1α,25 dihydroxy D_3 to the biologically inactive 1,24,25 trihydroxy D_3, Therefore a lower clinical dose of vitamin D may be effective against CRPC when it is combined with a clinically viable dose of androgen.

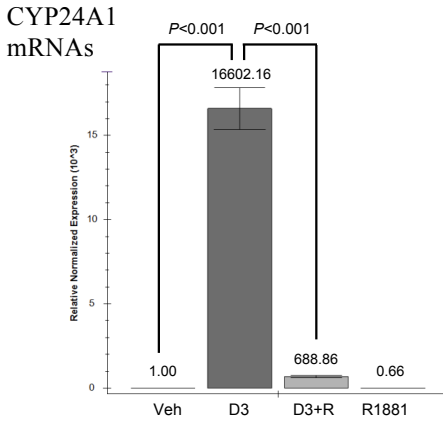
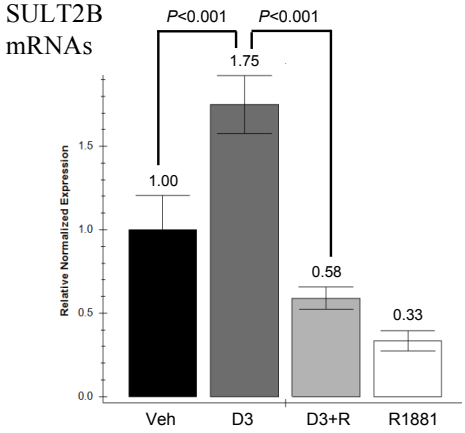


Fig. 3C. CYP17A1 mRNAs, normalized to β actin mRNAs from quantitative RT-PCR assay.

For SULT2B, however, its vitamin D-mediated induction was prevented when cells were treated concurrently with vitamin D and DHT (**Fig. 3D**). Nevertheless, this reduction of SULT2B expression should not influence CRPC growth, since the unconjugated DHEA pool itself is expected to decrease due to markedly lower expression, and thus activity, of CYP17A1 and HSD3β1 in response to concurrent exposure of cells to DHT and vitamin D.



Fig, 3D. SULT2B mRNAs, assayed by quantitative RT-PCR, and normalized to β-actin mRNAs.

In Vivo validation of the above data (Figs. 3A, 3B, 3C, 3D) in xenograft tumor settings is under way. Work in this section falls under the scope of Task 4 of approved SOW

IV ***LXRα-mediated induction of SULT2B***

We have reevaluated regulation of SULT2B expression by LXRα upon its activation by agonists and found that GW-3965, a synthetic LXR agonist, is superior to T0901317 (another synthetic LXR agonist) and β-sitosterol (a natural LXR agonist) in inducing SULT2B mRNA expression in human CRPC cells (C4-2, C4-2B, CWR22RV1 cell lines). We confirmed SULT2B induction at the protein level by western blot assay. We found that endogenous LXRα is expressed at much higher levels in CRPC cells (such as C4-2, C4-2B and CWR22RV1) compared to androgen-dependent prostate cancer cells (such as LNCaP, LNCaPII). These results provided background information that will facilitate our studies on i) induction of the LXR-SULT2B axis for growth reduction of CRPC tumors; ii) antagonism of AR activity by LXRα signaling and its impact on CRPC tumor growth.

Work in this section is in compliance with Task 3 and Task 4 of approved SOW.

Publications

Journal articles**:** None related to the DOD grant

Book chapter

1. Ahn J, Park S, Zuniga B, Bera A, Song CS, **Chatterjee B,** Vitamin D in prostate cancer (Litwack G, ed), Elsevier/Academic press, NY (Invited Chapter) (in press)— in print on February 1, 2016

2. Prakash CP*, Zuniga B*, Song CS*, Jiang S, Cropper JD, Park S, **Chatterjee B**. Nuclear receptors in drug metabolism, drug response and drug interactions. (Review) *Nuclear Receptor Research* (*equal contributors) (Revision submitted)

Conference presentation

1. Future submission of an abstract is planned on Nov 10 to the Annual Endocrine Society Meeting at Boston, April 2016
   Title: "*Vitamin D and Androgen Interplay in the Regulation of Androgen Biosynthesis and its Potential Impact on Castration-resistant Prostate Cancer*", by Sulgi Park, Jodie Cropper, Zhihua Zhang, Shoulei Jiang, Chung Song and Bandana Chatterjee

Impact:

On Primary Discipline

1. Findings described in section III are significant and can potentially have important impact; they may lead to successful intervention of CRPC with a combined regimen of androgen and $1,25D_3$, as explained above.
2. Findings described in section I suggest the possibility that reactivation of SULT2B expression may reduce CRPC tumor growth in human patients.

On Other Discipline

Vitamin D is known to inhibit other solid tumor cancers—most notably, colon cancer and breast cancer. Therefore, based on our results, hormonal combination where vitamin D is combined with other steroid hormones like estrogen, progesterone or dexamethasone may be beneficial against cancers beyond prostate cancer. This possibility should be explored.

On Technology Transfer

We have submitted a disclosure of our invention regarding the potential benefit of a combined therapeutic regimen of vitamin D and androgen against CRPC. We are waiting to hear for approval from the Office of Technology Commercialization (OTC) at our institution (UTHSCSA).

On society beyond science & technology

mCRPC is a non-curative terminal disease and a huge financial burden on affected families and on society. An overarching challenge in this regard is to develop new treatment strategies that circumvent resistance, which develops on current treatment protocols with new-generation anti-androgens (abiraterone acetate, a CYP17A1 inhibitor and enzalutamide, an AR antagonist). Our finding has the potential to unveil a new treatment approach that overcomes resistance to existing therapy. If successful, this will reduce hardship for patients and families of patients and lower the overall cost for healthcare management.

Changes/Problems

1. Technical problems with prostate tumor xenograft experiments using athymic nude mice, described under section IIc, need to be resolved. Utilization of NOD-SCID mice for the next experimental set should improve tumor take rate. Also, mice were subcutaneously injected at the left flank with control, SULT2B-intact cells and the same mouse received subcutaneous injection of SULT2B-silenced cells at the right flank. This strategy may have confounded rates of tumor growth for both left and right flank. For the next set of experiments, xenograft tumors will be produced with one type of cells in individual mice, which will receive either control cells (SULT2B-intact) or experimental cells (SULT2B-silenced).

2. A second problem was that we did not succeed gaining access to prostate cancer samples from African-American (AA) patients. We now understand that such samples are quite rare and not easy to get. We approached several different investigators requesting AA samples but were not granted access. We will contact North Carolina-Louisiana Prostate Cancer Consortium and the Canary Foundation Consortium (Palo Alto, CA) in search for TMAs with prostate cancer specimens from AA patients.

Products: An invention disclosure on results described under section III has been submitted to the Office of Technology Commercialization (OTC) at UTHSCSA. This disclosure, if approved by OTC, will protect our invention for one year from the date of approval of the disclosure.

Participants & Other Collaborating Organizations

A) Participants:

1) Name: Bandana Chatterjee, MSc, PhD, Professor

Project Role: PI
Researcher Identifier N/A

Nearest person month worked: 5 person months

Contribution to Project: **i)** Planned and designed experiments; **ii)** Initiated and coordinated collaboration with University of Washington (UW) on formalin-fixed mCRPC samples; **iii)** For SULT2B-IHC staining, worked with Institutional Pathology Core Service for staining of TMA samples (obtained from UW) and analysis of data with Dr. Sherry Werner (collaborator at UTHSCSA); **iv)** Supervised all assigned personnel; **v)** Held lab meetings; **vi)** Wrote & submitted two review chapters; revised manuscripts in order to address reviewers' comments; **vii)** Started preparing manuscripts for data obtained through the DOD project

Funding Support: DOD support: 10% through sub-award to UTHSCSA; Non-DOD support: i) VA salary for 87.5% appointment at VA through Research Career Scientist Award; ii) Morrison Trust Foundation (5%); iii) School of Medicine, UTHSCSA.

Please note: PI spent 25% efforts on the DOD project without any further compensation. Additional hours were also spent at nights, weekends.

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2) Name: Chung S Song, PhD, Assistant Professor

Project Role: PI for sub-award to UTHSCSA.
Researcher Identifier N/A

Nearest person month worked: 5

Contribution to Project: i) Conducted experiments on 1,25 vitamin D_3 ($1,25D_3$)-mediated gene regulation for enzymes involved in the intratumoral androgen biosynthesis in prostate cancer; **ii)** Cultured CRPC cells and prepared RNA samples for $1,25D_3$-treated cells in order to initiate global RNA sequencing of transcriptomes -- purpose is to identify additional players in the $1,25\text{-}D_3$-mediated regulation of genes associated with CRPC; **iii)** Discussed project direction with PI; Guided Ms. Sulgi Park and Ms. Jodie Cropper.

Funding Support: The sub-award from DOD covered 5% salary. Remaining salary covered by Merit-Review grant from VA awarded to PI.

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3) Name: Sulgi Park, MS

Project Role: PhD Student, working in PI's lab as a visiting student (Jan 2015-Jan 2017), officially enrolled in the PhD program at Yonsei Univ, Seoul, S Korea.
Researcher Identifier (e.g. ORCID ID): N/A

Nearest person month worked: 6

Contribution to Project: Ms. Park conducted experiments in two areas: 1) The role of SULT2B in the proliferation of castration-resistant prostate cancer cells *in vitro* and in xenograft tumors; 2) The role of 1,25-dihydroxy vitamin D_3 in the expression of the enzymes involved in androgen metabolism in CRPC cells; 3) Also, assisted in mouse experiments.

Funding Support: DOD support from April 2015-September 2015. Supported by Morrison Trust Foundation, San Antonio from Jan-March, 2015.

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4) Name: Ms. Jodie Cropper

Project Role: Research Assistant
Researcher Identifier N/A

Nearest person month worked: 11 months at 32 hrs/wk (Nov 2014-Sept 2015)
Contribution to Project: Ms. Cropper worked on **i)** the role of SULT2B in prostate cancer in xenograft tumors; absence of mycoplasma in cells that were used for experiments. **ii)** maintenance of prostate cancer cells for various experiments; **iii)** assays to confirm the absence of mycoplasma in cell culture.

Additionally, Ms. Cropper is the primary person to help PI on: **a)** negotiation with vendors for special quote price; **b)** placing purchase orders for lab supply, special reagents, mice; **c)** keep track of inventories on lab supplies/reagents for the DOD project.

Funding support: DOD grant (at 32 hrs/wk).

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5) Name: Sherry Werner, MD, Professor

Project Role: Co-investigator

Nearest person month worked: 0.3 person month

Contribution to project: **i)** Dr, Werner evaluated SULT2B IHC staining of TMA slides of mCRPC tissues, and took pictures of the slides at different magnifications. **ii)** PI, Dr. Chatterjee, has held meetings with Dr. Werner for experimental design and tissue acquisition for future studies. Dr. Werner is Co-director of genitourinary Tumor Bank at UTHSCSA.

Funding support: 5% of Dr. Werner's UT salary is covered by DOD sub-award. Dr. Werner is appointed 5/8th at VA and 3/8th at UTHSCSA. Rest is covered by institutional and NIH grant salary and from compensation due to clinical responsibilities and teaching.

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6) Name: Yidong Chen, PhD, Professor

Project Role: Collaborator

Nearest person month worked: 0.15 person month

Contribution to project: **i)** Calculated number of mice to include in each experimental group for statistical significance of results on animal experiments; **ii)** Bioinformatic analysis of transcriptome data on gene expression in CRPC cells treated with 1α,25-D_3 alone and with R1881 (synthetic androgen).

Funding support: 3% UT salary is covered by DOD sub-award. Additional salary is covered through various extramural grant support and by UTHSCSA.

B) Collaborating organizations

i) Elahe Mostaghel, MD, PhD, Associate Professor at University of Washington, Department of Medicine/Oncology & Fred Hutchison Cancer Center, Seattle: Through MTA, Dr. Mostaghel has provided us TMAs spotted with 70 formalin-fixed mCRPC tissues from distant metastases, recovered under a rapid autopsy program. We have examined SULT2B expression in these tissues by immunohistochemistry (IHC) using a polyclonal antibody raised in our laboratory.

ii) Dean A Troyer, MD, at the Eastern Virginia Medical School, Norfolk, VA: We received a limited number of formalin-fixed prostate cancer tissues as TMA slides from Dr. Troyer as part of our collaboration. These samples are being analyzed for SULT2B expression.

Special Reporting Requirements none

Appendices

1) Manuscript: Ahn et al, Vitamin D in Prostate Cancer, Chapter in *Vitamins and Hormones* (G. Litwack Ed), Volume 100, Elsevier/Academic Press, in press

2) Prakash et al, *Nuclear Receptor Research*, Review Article, (revision submitted)

3) Current Biosketch for PI

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME Chatterjee, Bandana

POSITION TITLE: Professor of Molecular Medicine

eRA COMMONS USER NAME (credential, e.g., agency login): chatterjeeb

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Calcutta, India	B.Sc.	1971	Chemistry (Honors)
University of Calcutta, India	M.Sc.	1973	Chemistry (Organic Chemistry specialization)
University of Nebraska, Lincoln, USA	Ph.D.	1977	Biochemistry
Oakland University, Rochester, MI	Postdoc	1977-80	Molecular Endocrinology

A. Personal Statement

My research expertise is in cell and molecular biology with a focus on molecular endocrinology. Our studies are pursuing new approaches to target therapy-resistant prostate cancer, which is a major cause of cancer deaths among men at the fifth decade and onward. We are following up on our two discoveries in the past several years -- **i)** methylation of androgen receptor (AR) at a single lysine residue by the SET9 methyl transferase and enhanced activity of methylated AR; **ii)** Loss of a steroid- and cholesterol-sulfating enzyme (SULT2B) in prostate cancer and its significance in enhanced de novo androgen biosynthesis in tumor cells. The results that agonists cognate to the vitamin D receptor (VDR) and liver X receptor (LXR) can induce SULT2B expression in prostate cancer cells suggest that induction of SULT2B by agonist-activated VDR and LXR may offer a new treatment strategy for metastatic castration-resistant prostate cancer (mCRPC). Our unpublished study, revealing a novel interplay between TORC1 and AR pathways, may help strategize a new approach to target mCRPC by repurposing old drugs.

Complementing above studies, I have collaborated with a virologist to examine NF-κB/cytokine- and interferon/Smad-regulated anti-cancer activity of the respiratory syncytial virus, RSV. Our data was the first evidence that RSV reduces growth of prostate tumor xenografts (a patent pending). CZ Biomed (Tampa, FL) has licensed this work. Clinical trials with RSV on CRPC patients are in the works.

My expertise in androgen receptor biology and in diverse methodologies, and my collaboration with clinician and basic scientists, is facilitating our prostate cancer research. My track record of leadership and accomplishments engender confidence that my funded research projects will be carried out successfully.

*Representative Publications***:**

1. Ahn J, Park S, Zuniga B, Bera A, Song CS and Chatterjee B, Vitamin D in Prostate Cancer, (Review Article) In: *Vitamins and Hormones* (G. Litwack Ed), Volume 100, in press, Elsevier/Academic Press,
2. Seo YK, Mirkheshti N, Song CS, Kim S, Dodd S, Ahn SC, Christy B, Mendez-Meza R, Ittmann MM, Werner SL, Chatterjee B. Sulfotrans-ferase SULT2B1b : Induction by vitamin D receptor, loss of expression in prostate cancer. *Mol. Endocrinology*, doi:10.1210/me.2012-1369**;** 27: 925–939, 2013.
3. Ko, S, Ahn J, Song CS, Kim S, Knapczyk-Stwora K, and Chatterjee B. Lysine Methylation and Functional Modulation of Androgen Receptor by Set9 Methyltransferase. *Mol. Endocrinology*, 25: 433-444, 2011.
4. Echchgadda, I, Chang, T-H, Sabbah, A, Bakri, I, Ikeno, Y, Hubbard, G, Chatterjee, B***** and Bose, S*. Oncolytic Targeting of Androgen-sensitive Prostate Tumor by the Respiratory Syncytial Virus (RSV): Consequence to Deficient Interferon-dependent Anti-viral Defense. *BMC Cancer*, 11:43-61, 2011. (* co-corresponding authors)
5. Shi, LH, Ko, S, Kim, S, Echchgadda, I, Oh, T, Song CS and Chatterjee B. Reciprocal Dynamics of the Tumor Suppressor p53 and poly(ADP-ribose)polymerase PARP-1 Regulates Loss of Androgen Receptor in Aging and Oxidative Stress, *J. Biol. Chem*. 283: 36474-36485, 2008.

B. Positions and Honors

1981-1987 Asst. & Assoc. Prof. of Biochemistry, Dept. of Chemistry, Oakland Univ. Rochester, MI
1986 Sandoz Visiting Scientist, Univ. of California, San Diego, CA
1988-1993 Assoc. Prof., Dept. Cellular & Structural Biology, UTHSC-San Antonio
1990- pres Res Health Scientist, S Texas Veterans Health Care System (STVHCS), San Antonio
1992-1999 Associate Research Career Scientist, Dept. Veterans Affairs, Washington, DC
1993-2004 Professor, Department of Cellular & Structural Biology, UTHSC-San Antonio
1999-2013 Sr Research Career Scientist Award, Dept. Veterans Affairs, Washington D.C.
2002- pres Professor of Molecular Med, Dept Molecular Medicine/Institute of Biotechnology, UTHSCSA
2002-pres Member, Barshop Institute of Longevity & Aging, San Antonio, UTHSCSA
2008-pres Faculty appointment, Masters of Science in Clinical Investigation (MSCI), UTHSCSA
2008-pres Member, Cancer Therapy and Research Center at UTHSCSA
2013-2018 Renewal Appt., Research Career Scientist, Dept. Veterans Affairs, Wash D.C.

HONORS:

2014 Elected AAAS Fellow**,** American Association for Advancement of Science (AAAS)
2014 Invited Speaker, Intnl Conference, Molecular Signaling in Health & Disease, Roorkee-IIT, India
2013 Research Career Scientist Award (Competitive Renewal), Dept. Veterans Affairs, Washington D.C.
2011 Golden Jubilee Plenary Speaker, North-East Institute Science & Technology (NEIST), Jorhat, India
2008 Cancer Scientist Award, Society of Asian-American Scientists in Cancer Research (SAASCR), USA
2002 Dean's Exceptional Graduate Teaching Excellence Award, UTHSCSA, San Antonio, Texas
1999 Sr Research Career Scientist Award, Dept. Veterans Affairs, Washington D.C.
1976 Franklin E. & Orinda M. Johnson Research Fellowship, University of Nebraska, Lincoln
1975 Maude Hammong Fling Fellowship, University of Nebraska, Lincoln
1973 Gold Medalist in Chemistry for Masters of Science (top-scoring graduate), Univ. Calcutta, India
1970 Gold Medalist in Chemistry for Bachelors of Science (top-scoring graduate), Univ. Calcutta, India

Advisory Committee Memberships (selected)

2014, 2015 Scientist Reviewer, UK Prostate Cancer, United Kingdom
2013 Member, Exploratory and Hypothesis Development Applications, DOD-PCRP, Dept. Defense
06-09;09-12 Member, Biology of Aging Review Committee, National Institute on Aging, NIH
2008 Panel Member, AACR Centennial Pre- and Post-doctoral Fellowship Application Review
'06-'09 Scientist Reviewer, US Army Prostate Cancer Research Program –DOD
2004-Pres Scientist Reviewer, James & Esther King Biomedical Res. Program (SOLIX), State of Florida
2003 Member, Special Panel (ZDK1GRB-6) Androgen Receptor, Prostate Growth, Cancer- NIDDK/NIH
2000 Member, Prostate Cancer Research Pgm, Massachusetts Public Health, Boston, MA
1999-2001 Member, Parent Research Committee, Am. Fed. Aging Research (AFAR), New York, NY
1997 Member, Special Review Panel, Reproductive Endocrinology Study Section, NIH
1994-1998 Member, Endocrinology Study Section, NIH

Editorial Service

2012-pres Academic Editor, *PLOS ONE*
2008-pres Associate Editor, *Endocrine Research*
2007-2009 Member, Editorial Board, *Molecular Endocrinology*
2003-2009 Member, Editorial Board, *American J. Physiology-Endocrinology Metabolism*
2009-pres Member, Editorial Board, *Am J. Phys-Endocrinology & Metabolism (renewed appointment)*
2000-2003 Member, Editorial Board, *Endocrinology*
2004 Guest Editor, Mech Aging Develop (Arun Roy Memorial Issue)
1984 Editor, Mol. Basis of Aging (Arun Roy and Bandana Chatterjee, eds.) Academic Press, NY
1988-pres Reviewer (ad hoc): *J Biol Chem; Mol Cell Biol; Oncogene; Nucl. Acid Res,* and 12 other journals

Membership to Professional Society

2006-pres American Association for Cancer Research (AACR)
1990-pres Endocrine Society
1982-pres American Society of Biochemistry and Molecular Biology
1983-pres American Association for Advancement of Science (AAAS)

Patents:

- Chatterjee B. Sulfotransferase and the Sulfation Pathway in Androgen Metabolism as Therapeutic Targets in Prostate Cancer (Provisional patent application filed in May), 2012.

- Bose, S, Chatterjee B: Use of Human Respiratory Syncitial Virus (RSV) in Killing Metastatic Prostate Cancer Cells in vitro, in vivo Mouse Tumor Xenograft: Therapeutic Potential. Filed 2008, (docketed/in que)
- Lavrovsky Y, Chen S, Song C, Chatterjee B, Roy AK: Estrogen Receptor Site-Specific Ribozymes Uses Thereof for Estrogen Dependent Tumors (UTSK 379US) US Patent No. 7,179,593; February 20, 2007
- Roy AK and Chatterjee B: Methods and Compositions Relating to the Androgen Receptor Gene and Uses Thereof. (UTSK:199) Patent No. 5,556,956; Date of Patent issued: September 17, 1996.

C. Contribution to Science

My research has centered around gene regulation by nuclear hormone receptors (NR) and its impact on health and disease. I initially studied androgen-regulated gene expression and then progressed to investigate androgen receptor gene and its regulation during aging and oxidative stress. My work was the first to establish that a transcription factor, namely androgen receptor, is differentially regulated during aging. I also explored roles of type-II nuclear receptors in steroid and drug metabolism. My lab provided the first evidence that vitamin D receptor (VDR) regulates phase II genes using SULT21A1 (DHEA/bile acid sulfotransferase) as a model. Currently, I am focused to decipher the role of androgen receptor and androgen signaling in prostate biology, especially in therapy (castration)-resistant prostate cancer and in prostate enlargement (BPH).

1. Early work: I studied androgen-induced, sexually dimorphic expression of a liver-secreted major urinary protein (α2u globulin), especially its modulation by growth hormone, thyroid hormone and insulin. Based on my work, α2u globulin emerged as an important model gene for studies on multi-hormonal regulation of a specific gene. Additionally, with an R01 support, my lab demonstrated for the first time that aging regulates expression of specific genes encoding α2u globulin and SULT2A1, and calorie restriction reverses age-associated differential expression of androgen receptor and androgen-regulated genes. From a historical perspective, this finding was significant, since it linked aging to gene regulation -- a concept not widely accepted by aging researchers in early eighties.

i) Chatterjee B, Roy AK: Messenger RNA for α2u-globulin. Purification, partial characterization of the mRNA, and synthesis of a Hae III restriction fragment as cDNA probe. ***J Biol Chem*.** 255: 11607-11613, 1980.
ii) Roy AK, Chatterjee B, Prasad MS, and Unakar NJ: Role of insulin in the regulation of the hepatic messenger RNA for α2u-globulin in diabetic rats. ***J Biol Chem*.** 255: 11614-11618, 1980.
iii) Chatterjee B, Nath TS, and Roy AK: Differential regulation of the messenger RNA for three major senescence marker proteins in male rat liver. ***J Biol Chem*.** 256: 5939-5941, 1981.
iv) Chatterjee B, Fernandes G, Yu BP, Song C, Kim JM, Demyan W, and Roy AK: Calorie restriction delays age-dependent loss in androgen responsiveness of the rat liver. ***FASEB J*.** 3: 169-173, 1989.
v) Song CS, Rao TR, Demyan WF, Mancini MA, Chatterjee B**,** and Roy AK: Androgen receptor messenger ribonucleic acid (mRNA) changes during maturation, aging, and calorie restriction. ***Endocrinology.*** 128: 349-356, 1991.

2. Aging, androgen receptor & transcription factors: We identified regulatory elements that direct loss of androgen receptor gene expression during aging. We showed that an age dependent factor (ADF) positively regulates rat androgen receptor gene, and ADF activity is greatly diminished with advancing age. We provided the first evidence that nuclear factor-κB (NF-κB) activity is upregulated during aging and NF-κB negatively regulates androgen receptor gene activity (*JBC, 1995*). These findings showed for the first time a mechanistic link between gene regulation and aging. Subsequently, my lab established ADF to be a coactivator complex comprised of the transcription factors B-Myb and c-Myb, poly ADP-ribose polymerase-1(PARP-1) and the RNA-binding hnRNP-K, which is a nucleoplasm-located ribonucleoprotein involved in mRNA metabolism.

i) Song CS, Her S, Slomczynska M, Choi SJ, Jung MH, Roy AK, and Chatterjee B**:** A distal activation domain is critical in the regulation of the rat androgen receptor gene promoter. ***Biochem J.*** 294: 779-784, 1993.
ii) Supakar PC, Song CS, Jung MH, Slomczynska MA, Kim JM, Vellanoweth RL, Chatterjee B**,** Roy AK: A novel element for age-dependent expression of rat androgen receptor gene. ***J Biol Chem.*** 268: 26400-26408, 1993.
iii) Supakar PC, Jung MH, Song C, Chatterjee B**,** and Roy AK: Nuclear factor κB represses rat androgen receptor gene; NF-κB activity increases with age-dependent desensitization of liver.***J Biol Chem*.** 270: 837-842, 1995.
iv) Chatterjee B, Song CS, Jung MH, Chen S, Walter CA, Herbert DC, Mancini MA, Roy AK: Targeted overexpression of androgen receptor with a liver-specific promoter in transgenic mice. ***Proc Natl Acad Sci USA*.** 93: 728-733, 1996.
v) Shi, LH, Ko, S, Kim, S, Echchgadda, I, Oh, T, Song CS and Chatterjee B. Reciprocal Dynamics of the Tumor Suppressor p53 and poly(ADP-ribose)polymerase PARP-1 Regulates Loss of Androgen Receptor in Aging and Oxidative Stress, ***J. Biol. Chem*.** 283: 36474-36485, 2008.

3. Nuclear receptors in steroid/drug metabolism: We showed that the phase II drug/steroid metabolizing gene encoding SULT2A1 is activated by the farnesoid X receptor (FXR), vitamin D receptor (VDR), pregnane X

receptor (PXR) and constitutive androstane receptor (CAR), and *SULT2A1* gene is repressed by androgen receptor. Since FXR regulates glucose, triglyceride and cholesterol metabolism in the liver, a role for FXR in the regulation of hepatic and intestinal SULT2A1 expression raises the possibility that beyond steroid/drug metabolism, SULT2A1 may regulate other liver/intestine-specific physiology and pathophysiology. Our study also provides significant new insights into drug-drug, drug-food and drug-herb interaction.

i) Song CS, Echchgadda I, Baek BS, Ahn SC, Oh T, Roy AK, and **Chatterjee B:** DHEA-sulfotransferase gene induction by bile acid activated farnesoid X receptor. ***J Biol Chem*** 276: 42549-42556, 2001.
ii) Chatterjee B, Echchgadda I, and Song CS: Vitamin D receptor regulation of the steroid/bile acid sulfotransferase SULT2A1. ***Methods Enzymol*** 400: 165-191, 2005.
iii) Song C S, Echchgadda I, Seo YK, Oh T, Kim S, Kim SA, Cho S, Shi L, and Chattterjee B An essential role of the CAAT/enhancer binding protein-alpha in the vitamin D-induced expression of the human steroid/bile acid-sulfotransferase (SULT2A1). ***Mol Endocrinol*** 20: 795-808, 2006.
iv) Echchgadda I, Song CS, Oh, T, De La Cruz J, Chatterjee B. Xenobiotic-sensing nuclear receptors PXR, CAR, orphan nuclear receptor HNF-4α in human steroid-/Bile Acid-Sulfotransferase. ***Mol. Endocrinol*** 21: 2099-2111, 2007.
v) Prakash CP, Zuniga B, Song CS, Cropper JD, Jiang S, and Chatterjee B. Nuclear Receptors in Drug-drug, Drug-food and Drug-herb interaction. *Nuclear Receptor Research* (Review Article)), (Revision submitted), 2015.

4. New approaches for t*argeting advanced prostate cancer*: We provided the first evidence for the cytoplasmic residency of androgen receptor (AR) in living cells in the absence of the cognate ligand androgen (*Mol*. *Endocrinol*, 2000). This was a significant result, since impaired nuclear translocation of AR is the basis for the activity of enzalutamide, a second generation AR antagonist which was developed later by Charles Sawyer's group and enzalutamide is used in the clinic against castration-resistant prostate cancer. My lab discovered that the level of the prostate expressed androgen-metabolizing sulfotransferase isozyme SULT2B is greatly reduced in human prostate cancer and in preclinical studies, prostate tumor growth accelerated upon loss of SULT2B in tumor cells. 1,25-dihydroxy vitamin D_3 (calcitriol) can induce SULT2B expression in prostate cancer cells, indicating that SULT2B may mediate some of the anticancer effects of vitamin D. We showed that androgen receptor activity is enhanced upon methylation by SET9 methyltransferase. This result links cellular nutrient states with AR function. Finally, we showed that the respiratory syncytial virus (RSV) showed oncolytic activity against androgen-dependent and castration-resistant prostate cancer, suggesting obvious therapeutic and potential of RSV. A US patent "*Use of Human Respiratory Syncitial Virus (RSV) in the Killing of Metastatic Prostate Cancer Cells in vitro, and in vivo in a Mouse Tumor Xenograft Model*", (co-inventors Bose & Chatterjee) is under review. CZ Biomed, at Tampa, Florida, has licensed this invention from my institution (University of Texas Health Science Center at San Antonio, UTHSCSA).

i) Tyagi RK, Lavrovski Y, Ahn S, Song C, Chatterjee B**,** Roy AK: Dynamics of intracellular movement and nucleocytoplasmic recycling of androgen receptor in living cells. ***Mol. Endocrinol***. 14: 1162-74, 2000.
ii) Ko, SY, Shi, LH, Song, CS and Chatterjee, B. Interplay of NF-κB and B-myb in TNFα-controlled negative regulation of androgen receptor expression. ***Mol. Endocrinol*** 22: 273-286, 2008.
iii) Kim K-Y, Yu SN, Lee SY, Chun S-K, Choi YL, Song CS, **Chatterjee B**, Ahn SC, Salinomycin- induced apoptosis of human prostate cancer cells due to accumulated reactive oxygen species and mitochondrial membrane depolarization. Biochim Biophys Res Commun., [Epub Aug 17, 2011], Sep 16; 413:80-86. 2011.
iv) Ko, S, Ahn J, Song CS, Kim S, Knapczyk-Stwora K, and Chatterjee B. Lysine Methylation and Functional Modulation of Androgen Receptor by Set9 Methyltransferase. ***Mol. Endocrinol*** 25: 433-444, 2011.
v) Echchgadda, I, Chang, T-H, Sabbah, A, Bakri, I, Ikeno, Y, Hubbard, G, Chatterjee, B and Bose, S. Oncolytic Targeting of Androgen-sensitive Prostate Tumor by the Respiratory Syncytial Virus (RSV): Consequence to Deficient Interferon-dependent Anti-viral Defense. ***BMC Cancer***, 11:43-61, 2011.
vi) Seo YK, Mirkheshti N, Song CS, Kim S, Dodd S, Ahn SC, Christy B, Mendez-Meza R, Ittmann MM, Werner SL, Chatterjee B. Sulfotrans-ferase SULT2B1b : Induction by vitamin D receptor, loss of expression in prostate cancer. ***Mol. Endocrinol***, 27: 925–939, 2013

Complete List of Published Work in My Bibliography is found from the link below:

http://www.ncbi.nlm.nih.gov/sites/myncbi/bandana.chatterjee.1/bibliograpahy/40336023/public/?sort=date&direction=ascending

D. Research Support

Active Support

- Department of Veterans Affairs – VA Merit Review -- 10/01/2013-09/30/2017 Role: PI

Title: "Inhtratumoral Androgen Receptor/Androgen Signaling in Prostate Cancer"

Examines regulation of intratumoral androgen metabolism by sulfotransferase (SULT2B) and efficacy of vitamin D receptor and liver X receptor in SULT2B induction and its exploitation for prostate cancer therapy.

- Dept. Veterans Affairs – VA Research Career Scientist (salary only) Chatterjee (PI) 04/01/13 – 04/30/18

Title: "Androgen Action in Prostate" Pursue research on prostate biology and pathobiology.

- DOD –Prostate Cancer Research Program 09/29/2014-09/28/2017 Role**:** PI

Title: "Role for a Steroid Sulfotransferase (SULT2B) in intratumoral androgen metabolism in prostate cancer"
Examines the specific role of the conjugative enzyme SULT2B in advanced prostate cancer

- Pilot Project --Morrison Trust Foundation, San Antonio: 10/01/2014-09/30/2015 Role; PI

Title: Aging, Benign Prostate Hyperplasia (BPH), Amelioration by Hormones and Phytonutrients
Examines molecular underpinnings of BPH and role of androgens in the disease

- Pilot (UTHSCSA)– President's Translational & Entrepreneurial Research Fund (PTEF) Role: Collaborator

Title: "Targeting Proteasomes to Treat Cancer"; 11/01/15-08/31/16; PI : Gaczynska M & Osmulsji P
Examines, in preclinical studies, the anticancer potency of a novel inhibitor of proteasomes.
Dr. Chatterjee's lab is responsible for all studies in mice.

<u>Completed Research Support</u> (past 5 years)

- Department of Veterans Affairs Senior Res Career Scientist 04/01/06 – 03/28/13 Role: PI

"Androgen Action in Prostate"; goal is to pursue the mechanism of prostate pathophysiology.

- 1I01BX000280-01 Chatterjee (PI) 10/01/09 – 09/30/13 Role: PI

Merit Review -- Department of Veterans Affairs
"Vitamin D3, Interference with Androgen Signal & Inhibition of Prostate Cancer"
Examines mechanism of inhibition of prostate tumor growth by vitamin D receptor (VDR).

- Biomedical Research Foundation, VA Werner (PI); Chatterjee (Co-PI) 09/01/12-08/30/14 Role: Co-PI

Diabetes in prostate cancer biology. This funded project is part of collaboration between Dr. Sherry Werner, M.D. and Dr. Bandana Chatterjee,Ph.D.

- CTRC Shared Resource Supplement Chatterjee (PI) 03/15/13 – 06/30/13 Role: PI

Androgen metabolism in prostate cancer tissue

- CTRC Shared Resource Supplement Chatterjee (PI) 03/15/13 – 06/30/13 Role: PI

Androgen metabolism in prostate cancer tissue

- Morrison Trust Foundation Chatterjee (PI) 10/01/09 – 09/30/10 Role: PI

"Vitamin D and Plant-derived Nutrients in the Amelioration of Prostate Cancer Progression"

- San Antonio Life Sciences Institute Bose (PI) 02/01/10 – 08/31/11 Role: Co-PI

(SALSI) Collaboration with UTSA Chatterjee (Co-PI)
"Microencapsulated delivery of respiratory syncytial virus for targeting prostate tumor"
--develop an optimal delivery of RSV for prostate cancer therapy.

- W81WXWH-08-1-0067 Ko (PI) 01/15/08 – 06/30/11 * Role: Mentor

DOD Prostate Cancer Res Progm (Pre-doc Fellowship) * Funds returned upon student graduation.
"Inflammation, Prostate Cancer and Negative Regulation of Androgen Receptor Expression"

- CTSA/UTHSCSA Translational Science Training Predoc Fellowship Ahn (PI) 8/1/09-8/31/10 Role: Mentor

- R21-CA129246-01A1 Bose, PI 05/01/08-04/30/10, NCE 04/30/2011 Role: co-PI

"Oncolytic Activity of Respiratory Syncytial Virus against Prostate Cancer"

Vitamins & Hormones, (Litwack G, ed), Elsevier/Academic Press (in Press)

Vitamin D in Prostate Cancer

Authors Jungmi Ahn[3], Sulgi Park[3], Baltazar Zuniga[2,3], Alakesh Bera[3], Chung Seog Song[3], and Bandana Chatterjee[1,3,¶]

Affiliations

[1]Research, South Texas Veterans Health Care System
Audie L Murphy VA Hospital, 7400 Merton Minter Boulevard
San Antonio, Texas 78229

[2]The University of Texas at Austin
2100 Comal Street, Austin, TX 78712

[3]Department of Molecular Medicine/Institute of Biotechnology
The University of Texas Health Science Center at San Antonio
Texas Research Park, 15355 Lambda Drive
San Antonio, Texas 78245

Key words: Vitamin D, vitamin D receptor (VDR), androgen receptor (AR), intracrine androgen metabolism, metastatic castration resistant prostate cancer (mCRPC), growth inhibition VDR metabolism, transcriptional regulation

¶Corresponding Author Bandana Chatterjee, Ph.D.
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E-mail: chatterjee@uthscsa.edu;

Tel:210-567-7218, FAX: 210-567-7222

Abbreviations NR, nuclear receptor; VDR, vitamin D receptor; AR, androgen receptor; DBD, DNA-binding domain; LBD, ligand-binding domain; NTD, N-terminal domain; CYP, cytochrome P450, 1α,25-D_3, 1α,25-dihydroxy vitamin D_3; mCRPC, metastatic castration resistant prostate cancer

Abstract

Metastatic castration-resistant prostate cancer (mCRPC) is a progressive, non-curable disease induced by androgen receptor (AR) upon its activation by tumor tissue androgen, which is generated from adrenal steroid DHEA through intracrine androgen biosynthesis. Inhibition of mCRPC and early-stage, androgen-dependent prostate cancer by calcitriol, the bioactive vitamin D_3 metabolite, is amply documented in cell culture and animal studies. However, clinical trials of calcitriol or synthetic analogs are inconclusive, although encouraging results have recently emerged from pilot studies showing efficacy of a safe-dose vitamin D_3 supplementation in reducing tumor tissue inflammation and progression of low-grade prostate cancer. Vitamin D-mediated inhibition of normal and malignant prostate cells is caused by diverse mechanisms including G1/S cell cycle arrest, apoptosis, pro-differentiation gene expression changes and suppressed angiogenesis and cell migration. Biological effects of vitamin D are mediated by altered expression of a gene network regulated by the vitamin D receptor (VDR), which is a multi-domain, ligand-inducible transcription factor similar to AR and other nuclear receptors. AR-VDR crosstalk modulates androgen metabolism in prostate cancer cells. Androgen inhibits vitamin D-mediated induction of CYP24A1, the calcitriol-degrading enzyme, while vitamin D promotes androgen inactivation by inducing phase I monooxygenases (e.g. CYP3A4) and phase II transferases (e.g. SULT2B1b, a DHEA-sulfotransferase). CYP3A4 and SULT2B1b levels are markedly reduced and CYP24A1 is overexpressed in advanced prostate cancer. In future trials, combining low-calcemic, potent next-generation calcitriol analogs with CYP24A1 inhibition or androgen supplementation, or cancer stem cell suppression by a phytonutrient such as sulforaphane, may prove fruitful in prostate cancer prevention and treatment.

1. Introduction

Biologically active vitamin D known as calcitriol (1α, 25-dihydroxy vitamin D_3) is a secosteroid, best characterized for its essential endocrine role in bone mineralization, which is a consequence of the regulation of calcium and phosphate homeostasis by this hormone (DeLuca, 2014; Feldman et al, 2014). Vitamin D deficiency causes softening of bone from insufficient mineralization, which manifests as rickets in children and osteomalacia in adults. Beyond bone health, a broad range of physiological processes including inflammation, angiogenesis, apoptosis, differentiation and cell growth and proliferation are influenced by vitamin D's autocrine and paracrine actions on extraskeletal tissues. The anti-proliferative effect of calcitriol has been demonstrated in cell culture and *in vivo* in animal models of various cancers including colon, breast and prostate cancer (Deeb et al, 2007; Feldman et al, 2014). Cell growth inhibition of normal and malignant prostate cells by vitamin D has been linked to diverse mechanisms including G1→S cell cycle arrest, DNA damage reduction, microRNA regulation, apoptosis induction and pro-differentiation changes. Calcitriol inhibits primary prostate cancer cells isolated from clinical specimens, cell lines from prostate cancer, and tumor xenografts of prostate cancer. However, vitamin D's clinical benefit is uncertain, since a high serum vitamin D status is weakly linked to reduced prostate cancer risk in epidemiologic research, and clinical trials of vitamin D are inconclusive (Feldman et al, 2014). Reduction of tumor burden by vitamin D alone is untenable since calcitriol or its analog at a supra-physiologic clinical dose induces hypercalcemia, which elevates risks for cardiovascular disease and lethal prostate cancer (Okamoto et al, 2012, Datta & Schwartz, 2012). Nevertheless, a recent pilot study shows that progression of low-grade prostate cancer is prevented or even reversed by long-term, safe-dose vitamin D supplementation (Marshall et al, 2012).

Genomic action of vitamin D is mediated by the cognate nuclear vitamin D receptor (VDR), which is a ligand-inducible transcription factor (Haussler et al, 2011; Carlberg, 2014; Pike et al, 2014). The ligand-activated VDR, in association with coregulators, mediates transcriptional induction or

repression of VDR/vitamin D target genes. Rapid, non-genomic vitamin D action (induced within several minutes) involving a membrane-bound VDR, which is unrelated to the nuclear VDR, has also been described (Haussler et al, 2011). Calcitriol can reduce DNA damage from thymine dimers caused by UV radiation, thus providing protection against sunlight-induced skin cancer. This DNA damage regulation involves non-genomic vitamin D action and requires interaction of the endoplasmic reticulum stress protein 57 (ERP57) with the membrane-bound VDR (Sequeira et al, 2012).

The androgen receptor (AR) plays a central role in prostate cancer development and progression. Adenocarcinoma of the prostate gland is a leading cause of cancer death in men worldwide, and other than skin cancer it is the most frequently diagnosed cancer in males of the Western society. American Cancer Society estimates that in 2015 prostate cancer will claim roughly 27,500 lives in the USA and more than 220,000 new cases will be diagnosed. Prostate is also the primary site for sarcoma and carcinomas of neuroendocrine cells, small cells, transitional cells which, unlike adenocarcinoma, are extremely rare. Radiation and radical prostatectomy, individually or in combination, is the standard-of-care for gland-localized primary prostate cancer, and androgen deprivation therapy (ADT), which induces apoptosis for androgen-dependent prostate cancer cells, is used against locally invasive prostate cancer. ADT, which entails depletion of serum androgen to a castrate level via pharmacologic or surgical intervention, may be combined with AR antagonists (such as Casodex) for complete blockade of the androgen axis. A recent report on ~900 patients receiving ADT shows that for about 70% patients, cancer progressed within 20 months (Harshman et al, 2015). An overarching problem in prostate cancer management is that due to the lack of suitable biological and pathological markers, indolent tumors cannot be distinguished from aggressive tumors at initial diagnosis.

Metastatic castration-resistant prostate cancer (mCRPC), a non-curable terminal condition, is primarily driven by restored AR activity. Reactivation of AR occurs by several mechanisms -- most

prominently due to AR overexpression, ligand-independent activity of AR splice variants and intracrine production of testosterone and 5α-dihydrotestosterone (androgens) *de novo* in tumor tissue (Sher et al, 2004; Titus et al, 2005; Mostaghel & Nelson, 2008; Mostaghel et al, 2010). Inhibition of reactivated AR accounts for the efficacy of second-generation AR antagonists (such as enzalutamide) and androgen biosynthesis blockers (such as abiraterone acetate) in inhibiting mCRPC progression for additional 4 to 5 months (Tran et al, 2009; de Bono et al, 2011; Mostaghel EA, 2014). Crosstalk of the VDR and AR pathway contributes to prostate cancer inhibition by vitamin D in experimental models (Wang & Tenniswood, 2014). Detailed insights into the interplay of these two nuclear receptor pathways in castration-resistant prostate cancer are expected to identify novel approaches for controlling mCRPC.

This review covers an overview of i) enzymes directing vitamin D biosynthesis and degradation and their relevance to prostate cancer; ii) VDR as a ligand-inducible transcription factor, its functional domains, its recognition of vitamin D-responsive DNA element (VDRE) and domain-induced allostery; iii) vitamin D-mediated regulation of cellular processes relevant to tumor growth inhibition; iv) preclinical studies and clinical trials of calcitriol and analogs; v) tumor tissue VDR levels and risks of lethal prostate cancer. Finally, vitamin D action in the context of VDR-AR crosstalk and intracrine androgen metabolism in prostate cancer will be discussed.

2. VITAMIN D METABOLISM: SYNTHESIS, DEGRADATION, RELEVANCE TO PROSTATE CANCER

Vitamin D refers to molecules with a steroid-like structure (secosteroid) that serve as prohormones for the bioactive hormone calcitriol. Vitamin D_2 (ergocalciferol) produced in plants and vitamin D_3 (cholecalciferol), synthesized in the skin exposed to sun's ultraviolet radiation, are the two physiologically relevant vitamin D forms in humans, D_2 being a weaker prohormone than D_3. Besides natural production, dietary products (D_3-fortified dairy products, fatty fish, fish liver oil and eggs) and

D_3 supplement are the other two sources for D_3 intake. The vitamin D status of an individual is indicated by serum levels of the 25-hydroxy-D_2/D_3 (collectively 25(OH) D), which are produced predominantly in the liver by enzymatic hydroxylation of D_2 and D_3. Bioactivation of the circulating 25-hydroxy D to the potent hormone $1\alpha,25(OH)_2D_3$ (specified as calcitriol) entails additional hydroxylation at the carbon-1 position. Calcitriol biosynthesis occurs predominantly in the kidneys, although production of $1\alpha,25(OH)_2D_3$ can also occur in a number of extrarenal tissues including prostate (DeLuca, 2014; Feldman et al, 2014).

Vitamin D deficiency is highly prevalent among African Americans and higher incidences of aggressive prostate cancer and higher mortality rates from this malignancy are observed in men from this race group. Epidemiologic data in some examples has shown an inverse correlation between serum vitamin D and prostate cancer risks, although the data is inconsistent with several other population-based studies (Li et al, 2007; Feldman et al, 2014). Current thinking is that an intermediate range of vitamin D status may be optimal—both high and low serum vitamin D may be adversely linked to prostate cancer risks and prostate cancer progression (Albanes et al, 2011). The serum 25(OH) vitamin D level at 50-70 ng/ml range is recommended for the benefit of bone health; whether the same level of serum vitamin D would be beneficial against prostate cancer risks has not been settled (Feldman, 2014).

2.1 Enzymatic machinery for Vitamin D biosynthesis and degradation

Vitamin D synthesis in skin starts from the conversion of 7-dehydrocholesterol to previtamin D_3 by UVB at the 282–310 nm radiation of the solar energy spectrum. Pre-vitamin D_3 undergoes temperature-dependent isomerization to vitamin D_3. As noted above, vitamin D_3 is sequentially hydroxylated first to 25-hydroxy D_3 in the liver by the microsomal CYP2R1 (Zhu et al, 2013) and next, in the kidney proximal tubule, to the active metabolite 1α, 25-dihydroxy vitamin D_3 (abbreviated 1, 25-D_3) by the

mitochondrial CYP27B1 (DeLuca, 2014). Figure 1 schematically shows biosynthesis of functional vitamin D, its degradation by CYP24A1 and its auto-regulation and regulation by other factors.

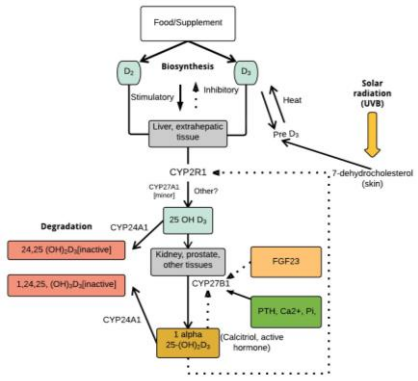

Figure 1 Enzymatic pathway for calcitriol biosynthesis, degradation and regulation

An inherited loss-of-function mutation of CYP2R1 is associated with low circulating vitamin D and classic symptoms of vitamin D deficiency (Al Mutair et al, 2012). A critical role of CYP27B1 in vitamin D action is evident from patients carrying loss-of-function or reduced function mutations of this enzyme. Null inactivated or reduced CYP27B1 activity causes vitamin D-dependent rickets-type1, an autosomal recessive disease exhibiting hypocalcemia, hypophosphatemia and consequent development of fracture-prone soft, weak bone and bowed legs (Babiker et al, 2014). CYP27B1 expression is transcriptionally upregulated by parathyroid hormone, calcium, phosphorous and downregulated by 1, 25-D_3. CYP27B1 expression is also suppressed by the bone-derived circulating peptide FGF23, which regulates parathyroid hormone secretion (Chanakul et al, 2013). CYP24A1, a mitochondrial 1, 25-dihydroxyvitamin D_3 24-hydroxylase (24-hydroxylase), inactivates calcitriol by converting it to a carbon-24 hydroxylated metabolite. Robust induction of CYP24A1 by calcitriol prevents excessive accumulation of this hormone in target tissues and helps maintain vitamin D homeostasis.

2.2 Vitamin D metabolism in prostate cancer

Prostate cancer cells and prostate tumor tissue express the enzymatic machineries for supporting local synthesis of calcitriol (Chen & Holick, 2003; Chen, 2008; Chen et al, 2012; Feldman et al, 2014). The activity of CYP27B1, the major mediator of 25(OH)D bioactivation to calcitriol, is repressed in prostate

cancer cells in culture and in prostate tumor tissue, in contrast to the relative abundance of this enzyme in the non-malignant prostate epithelium (Hsu et al, 2001). Epidermal growth factor (EGF) can upregulate *CYP27B1* gene transcription in non-malignant prostate epithelial cells, but not in prostate cancer cells (Jamieson et al, 2004). It has been suggested that CYP27B1 plays a role in the normal regulation of prostate cell growth and dysregulation of CYP27B1 may, in part, contribute to the uncontrolled cell growth and proliferation in prostate cancer (Hsu et al, 2001). Nevertheless, a recent study concluded that cholecalciferol and calcitriol were equally effective in reducing tumor growth in a prostate cancer xenograft model (Swami et al, 2012). This observation indicates that 25-hydroxy-vitamin D_3-1α-hydroxylase activity is present in prostate cancer tissue.

Homeostasis of the vitamin D hormone in prostate is impacted by CYP24A1 expression. Since calcitriol strongly induces CYP24A1, resistance to vitamin D therapy may occur from elevated CYP24A1 expression. In fact, increased CYP24A1 expression correlated with advanced stages of prostate cancer (Tannour-Louet et al, 2014). Thus, combined targeting of prostate tumor by inhibiting CYP24A1 with a small-molecule inhibitor and activating VDR signaling by vitamin D_3 supplementation is potentially a fruitful avenue for therapeutic intervention. Chronic lymphocytic leukemia cells were inhibited when co-treated with a CYP24A1-specific small-molecule inhibitor (such as an imidazole styrylbenzamide) and calcitriol, which induced *GADD45α* (encoding growth arrest and DNA damage inducible protein) and *CDKN1A* (encoding the cell cycle inhibitor p21) (Ferla et al, 2014).

Homeostasis of active vitamin D is also impacted by its phase I oxidation mediated by CYP3A4 and by phase II glucuronidation mediated by 5'-diphosphoglucurosyltransferase (UGT). UGT1A4 and UGT1A3 are involved in the glucuronidation of 25(OH)D_3. Glucuronide conjugates of 25(OH)D_3 have been detected in human plasma and bile (Wang et al, 2014). Phase I/phase II modifications, occurring primarily in the liver and intestine, facilitate vitamin D clearance from the body. Since CYP3A4 and

UGTs are induced by drug-activated nuclear receptors PXR (pregnane X receptor) and CAR (constitutive androstane receptor) (Aleksunes & Klaassen, 2012), prescription drugs and other xenobiotic factors may reduce the serum vitamin D level, which in turn would reduce the availability of vitamin D as an anti-cancer agent. These confounders should be considered in order to correctly evaluate the efficacy of vitamin D supplementation for chemoprevention and its role as a cancer therapeutic.

3. VDR-REGULATED GENE TRANSCRIPTION: LIGAND SPECIFICITY, DNA RESPONSE ELEMENTS, DOMAIN-INDUCED ALLOSTERY

Vitamin D receptor (VDR) is a ligand-inducible transcription factor and a member of the nuclear receptor (NR) superfamily, which in the case of humans includes 48 receptors. NR proteins, which regulate nearly all aspects of the physiological processes necessary to create and support life, are defined by a common structural organization containing multiple functional domains (Evans & Mangelsdorf, 2014; Carlberg, 2014; Helsen & Claessen, 2014). VDR (NR1I1) has a short (24 amino acids), unstructured amino-terminal domain (NTD), a central DNA-binding domain (DBD) containing two helical zinc finger modules and a carboxyl-terminal ligand-binding domain (LBD). A mostly unstructured hinge domain connecting DBD and LBD allows for structural flexibility of the DNA-bound VDR and its partner RXR. Structural determination of VDR and other NRs by X-ray crystallography and cryo electron microscopy and in solution, by small-angle X-ray scattering (SAXS), small-angle neutrino scattering (SANS) and hydrogen-deuterium exchange revealed conformational details for each functional domain and for the full-length receptor in the ligand-bound, DNA-associated form (Huang et al, 2010; Zhang et al, 2011; Carlberg & Campbell, 2013; Watson et al, 2013).

Nucleus-residing unliganded VDR remains in a chromatin-bound repressed state similar to what was first demonstrated for two other NRs, namely thyroid hormone receptor (TR) and retinoic acid receptor (RAR) (Rosenfeld et al, 2006). The unliganded VDR recruits corepressors (such as NCoR1,

SMRT/NCoR2), which in turn recruit a histone deacetylase (HDAC) complex -- the net effect being generation of a VDR-containing compact chromatin region and gene repression. As an example, our study showed that unliganded VDR suppressed the basal CYP24A1 level in breast cancer cells, and VDR silencing (by siRNA) elevated the basal level of CYP24A1 (Alimirah et al, 2010). The ligand-dependent activation phase of VDR is initiated when change in LBD conformation due to the binding of 1,25-D_3 at the ligand-binding pocket and repositioning of LBD helix-12 create an interaction surface for coregulator exchange, replacing corepressors with coactivators (VaÈisaÈnen et al, 2002). Binding of a pioneering factor is thought to open the chromatin region and facilitate VDR binding (Carlberg, 2014). The AF2 activation function at LBD for VDR (and all other NRs) arises from coactivator recruitment to LBD induced by repositioned helix-12. A coactivator of the p160 family (SRC-1/-2/-3) makes physical contact with VDR via an LXXLL motif and also recruits histone acetyltransferases (such as CBP/p300, p/CAF), which mediate acetylation at specific lysine and arginine residues of histone H3 and H4. Chromatin relaxation due to histone acetylation sets in motion the assembly of other classes of coregulator complex including additional histone modifiers (methyltransferase/demethylase, ubiquitin ligase/deubiquitinase, kinase/phosphatase), chromatin remodelers (such as SWI/SNF containing WINAC complex, Rosenfeld et al, 2006). DRIP, a VDR-interacting mediator complex, functionally couples VDR-associated co-activators to the regulatory machinery at the transcription start site (TSS), leading to increased RNA polymerase II (Pol II) activity and gene induction. VDREs in *CYP24A1* have been characterized (Luo et al, 2010; Carlberg, 2014). CYP24A1 mRNAs were induced more than 2000-fold when castration-resistant C4-2B prostate cancer cells were treated with EB1089 (Fig. 4).

VDR mediates gene repression through a direct mechanism known as transrepression (Rosenfeld et al, 2006). Reduced *CYP27B1* gene expression by 1,25-D_3 in the kidneys entails association of the ligand-bound VDR with several negative vitamin D response elements (nVDRE) (Turunen et al, 2007). The *CYP27B1* upstream promoter contains two types of nVDREs -- one having sequence organization

resembling a positive VDRE with specific affinity for the VDR/RXR dimer in a ligand-dependent manner, and the other with binding specificity for a specific transcription factor (TF), such as VDIR, whose activity is inhibited by tethered VDR or by nVDRE-bound VDR. The latter example of nVDRE bears no similarity to a classical VDRE (Turunen et al, 2007).

3.1 DNA Response Elements

VDR binds a vitamin D response element (VDRE) as a dimer in association with a partner protein. Retinoid X receptor (RXR), the NR that binds to 9-cis retinoic acid is the partner for dimerization in majority of cases. Nevertheless, another DNA-bound transcription factor may also be the dimer partner for VDR as noted above (Carlberg et al, 1993). Protein-protein interaction at the interface of VDR and RXR DBDs stabilizes the dimer, with further stabilization arising from the interaction of LBDs of VDR and RXR. Dimerization stabilizes VDR-DNA interaction by circumventing the otherwise low DNA-binding affinity of a VDR monomer (Helsen & Claessen, 2014). The classical VDRE for the VDR/RXR complex contains variations of the sequence 5'-AG(G/T)TCA (consensus half-site sequence), configured as a direct repeat (DR) with variable number of spacer nucleotides (DR-n) separating the AG(G/T)TCA repeat. VDR contacts the 6-base 5' half-site within the major groove of DNA via the first zinc finger module of DBD (Carlberg & Campbell, 2013). The second zinc finger of VDR associates with the RXR DBD. A short c-terminal extension after the second zinc finger further stabilizes receptor-DNA interaction (Helsen & Claessens, 2014). Genome-wide analysis identified DR3 as the preferred response element for VDR in a chromatin context (Tuoresmaki et al, 2014). A DR3 element mediates robust VDR-mediated transactivation of *CYP24A1* by 1,25-D_3 (Carlberg & Campbell, 2013). Other DR configurations (most frequently DR4, DR5) are also involved in vitamin D-mediated induction of target genes. A DR4-type nVDRE mediates transrepression of *CCNC*, encoding cyclin C (Carlberg & Campbell, 2013). In our study, we reported the involvement of a DR7-type VDRE in the VDR-mediated induction of *SULT2B1* sulfotransferase in prostate cancer cells (**Fig. 2A**; Seo et al,

2013). SULT2B1b (hereon referred as SULT2B) is a prostate-expressed cholesterol- and DHEA-sulfotransferase encoded by *SULT2B1*. SULT2B levels are reduced in primary prostate cancer and its expression is non-detectable in >90% cases of distant metastasis that we have analyzed (Fig. 3). Consistent with its transactivation role, we found cyan fluorescent protein labeled VDR (CFP-VDR) localized mostly in the nucleus of calcitriol-treated COS-1 cells (monkey kidney derived) that expressed transfected CFP-VDR. In the absence of the ligand, both nuclear and cytoplasmic compartment showed fluorescence indicating nucleocytoplasmic distribution of the receptor **(Fig. 2B)**. The subcellular localization is tissue- and cell type-specific, since in MCF-7 breast cancer cells, unliganded VDR resides in the nucleus (Alimirah et al, 2010).

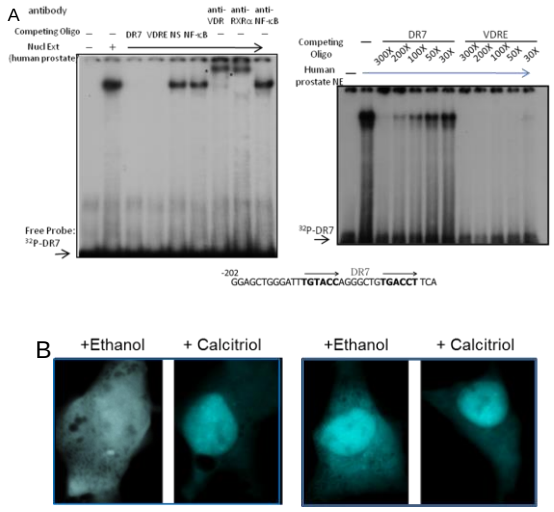

Figure 2 A: EMSA complex of a DR7-type DNA element in the *SULT2B1* promoter to proteins in the nuclear extract of normal human prostate, competition of a well-characterized DR3-type VDRE from the osteocalcin promoter for binding to the EMSA complex, and antibody supershift assay showing the presence of VDR and RXR-α in the EMSA complex. (data taken from Seo et al, 2013)

Figure 2B: Subcellular localization of transfected CFP-VDR in COS1 cells treated with ethanol or calcitriol. Images from two separate sets of experiments are shown

3.2 Domain-induced allostery

VDR-regulated gene activity can be influenced by the chemical structure of the VDR agonist, nucleotide sequence of the DNA response element and surface topography of NTD -- each specifying domain-dependent allostery. Functional crosstalk among various domains of VDR due to allosteric interaction was revealed by structural and biochemical analyses (Meijsing et al, 2009; Huang et al, 2010; Zhang et al 2011,; Watson et al, 2013). Relay of inter-domain signal for the VDR/RXR complex was evident from hydrogen-deuterium exchange profiling of the heterodimer. Ligand binding to VDR increased solvent exchange at its DBD indicating that ligand occupancy and the ensuing conformational

change of LBD impacted DBD conformation and thus, its affinity for the cognate element (Zhang et al, 2011). This conformational change would potentially alter gene expression.

Influence of the ligand structure on target gene expression is revealed from differential effect of the natural (1,25-D_3) versus synthetic (EB1089) ligand of VDR on the transactivation of androgen metabolism genes in prostate cancer cells (Doherty et al, 2014). The mRNAs of enzymes such as CYP3A4, CYP3A5, AKR1C3 were induced strongly by EB1089 but not by calcitriol in androgen-dependent prostate cancer cells. On the other hand, calcitriol, not EB1089, induced mRNAs for 17β-hydroxysteroid dehydrogenase-2 and prostaglandin dehydrogenase (Doherty et al, 2014).

Signal transmission from DBD to LBD for DNA-bound VDR has been demonstrated as well (Zhang et al, 2011). Receptor binding to a DR3 element altered the LBD surface for coactivator contact that led to altered SRC1 interaction with VDR and with the VDR/RXR complex (Zhang et al, 2011). An example of NTD-DBD communication comes from our finding, which showed that a polymorphic site (FokI-FF) at the VDR NTD, which deletes 3 amino acids from the naturally occurring VDR variant, abolished repression of the *CYP24A1* promoter by unliganded VDR (Alimirah et al, 2010).

4. INHIBITION OF PROSTATE CANCER BY VITAMIN D: INSIGHTS FROM CELL CULTURE AND PRECLINICAL STUDIES, AND CLINICAL TRIALS

Growth inhibitory effects of ligand-activated VDR on normal and malignant epithelial cells from diverse tissues including colon, breast and prostate have been extensively investigated (Getzenberg et al, 1997; Deeb et al, 2007; Salehi-Tabar et al, 2012; Feldman et al, 2014). Impaired cell growth from vitamin D/VDR action is driven by several distinct mechanisms, such as cell accumulation at G0-G1 and consequent G1→S cell cycle arrest; induction of differentiation, apoptosis, and inhibition of angiogenesis, cell adhesion and cell migration (Sung & Feldman, 2000; Deeb et al, 2007). Calcitriol

inhibits proliferation of normal prostate epithelial cell line, cells of primary culture from normal and cancerous prostate tissue and prostate cancer cell lines with an androgen-dependent or androgen-independent phenotype. Cells from normal prostate epithelium are more sensitive (~ by two orders of magnitude) to 1,25-D_3 mediated proliferation inhibition than cells originating from prostate cancer. Higher corepressor levels (NCoR1, NCoR2/SMRT) and lower levels of VDR in prostate cancer cells compared to normal prostate epithelial cells may partly account for differential sensitivity of normal versus cancerous prostate cells to vitamin D-induced inhibition. Furthermore, less aggressive prostate cancer cells (such as androgen-dependent LNCaP cells) are more sensitive to the anti-proliferative action of calcitriol than more aggressive prostate cancer cells such as PC3 and DU145. Mechanisms for growth inhibition are diverse and cell-type dependent. G1→S arrest and apoptosis are induced in androgen-responsive, moderately differentiated LNCaP prostate cancer cells upon treatment with active vitamin D or analogs; but no G0-G1 accumulation occurs for calcitriol-treated PC3 cells and these cells were much less inhibited for proliferation by calcitriol than LNCaP cells (Zhuang & Burnstein, 1998). Calcitriol-treated PC3 and DU145 cells exhibited reduced cell adhesion, migration and invasion (Sung & Feldman, 2000), and growth factor stimulated proliferation and invasion of DU145 cells was inhibited by BXL-628, a calcitriol analog (Marchiani et al, 2006). Disparate expression/activity of CYP24A1 and resulting differences in the prostate level of the active vitamin D_3 metabolite also play a role in cell-type dependent variations of the anti-proliferative response. Reduced metabolism of 1,25-D_3 in the presence of a CYP24A1 inhibitor elevated VDR signaling in DU145 cells (Yee et al, 2006). Clinical resistance to vitamin D therapy may result from augmented metabolism of 1,25-D_3 since *CYP24A1* gene amplification and its enhanced expression in clinical prostate cancer specimens have been reported (Tannour-Louet et al, 2014). Growth-promoting pathways that are known to be inhibited by VDR signaling in prostate cancer cells are described below (Deeb et al, 2007)

4.1. Mechanisms for anti-proliferative actions

a) ***Cell cycle arrest***

Inhibition of G1→S cell cycle progression in VDR-activated non-malignant and malignant prostate epithelial cells is associated with elevated expression of p21/Cip1, p27/Kip1, and p15/INK4b, which are cyclin-dependent kinase inhibitors (CKIs) (Campbell et al, 1997; Zhuang & Burnstein, 1998; Moffatt et al, 2001; Boyle et al, 2001; Yang & Burnstein, 2003) A well-documented consequence of p21 up-regulation in calcitriol-treated prostate cancer cells is hypophosphorylation of the retinoblastoma protein pRb that results in the sequestration of E2F transcription factors and inhibition of E2F target genes that are involved in DNA synthesis, thereby blocking S-phase entry of cells. VDR-regulated overexpression of *CDKN1A* (encoding p21) in RWPE-1 non-malignant prostate epithelial cells is epigenetically controlled by histone H3 acetylation at lysine-9 (Thorne et al, NAR, 2011). *CDKN1A* induction is also associated with stress-induced G1/S cell cycle arrest; in this case, transactivation of *CDKN1A* is mediated by p53, which is a transcription factor and tumor suppressor.

Induction of *CDKN2B* (encoding p15/INK4b, a CKI of the INK4 family) in normal prostate epithelial cells in response to calcitriol is an indirect effect caused by VDR-stimulated TGFβ1-Smad signaling (Robson et al 1999). Activities of cyclin D bound CDK4 and CDK6 are inhibited by p15/INK4b (Sherr & Roberts, 1999), which leads to pRb accumulation in a hypophosphorylated form with a net result of G1→S block due to E2F sequestration.

Reduction of the cMYC oncoprotein level, and consequent hypophosphorylated pRb and pRb-mediated E2F sequestration, is another mechanism for vitamin D/VDR mediated G1→S cell cycle block. Calcitriol-mediated reduction of the cMYC protein level has been reported for non-malignant prostate cells (RWPE-1), androgen-dependent prostate cancer cells (LNCaP, LAPC4, VCaP) and for androgen-independent C4-2 prostate cancer cells (Rohan & Weigel, 2009; Washington & Weigel, 2010; Salehi-Tabar et al, 2012). cMYC down regulation in this case is a result of reduced cMYC mRNA expression along with destabilization of the c-MYC protein (Rohan & Weigel, 2009).

In our study, we observed E2F1 mRNA and protein levels were reduced in calcitriol-treated LNCaP cells and a decline in E2F1 promoter activity accounts for this reduction. A calcitriol-responsive DNA element in the upstream E2F1 promoter binds several regulatory proteins (Ahn et al, manuscript in preparation). Reduction of E2F1 in LNCaP cells in response to calcitriol is consistent with what was reported earlier (Zhuang & Burnstein, 1998; Rao et al, Cancer Res 2004).

VDR-induced p27/Kip1 accumulation in LNCaP cells caused a reduced nuclear CDK2 level (due to extrusion of nuclear CDK2 to the cytoplasm) – the net consequence being inactivation of the cyclin E/CDK2 complex and calcitriol-mediated inhibition of the S-phase entry of LNCaP cells (Yang & Burnstein, 2003). Additionally, p27 was found to be stabilized due to inhibition of its proteasomal degradation caused by reduced p27 phosphorylation at threonine-187, and reduction of the level of Skp2, which is a component of the SCF ubiquitin ligase complex.

Calcitriol may also induce cell cycle arrest by increasing the expression of GADD45α, which is a stress-induced protein. DNA-damaging agents including docetaxel, a chemotherapeutic, induce *GADD45α*. GADD45α disrupts the CDK1/cyclinB1 complex by binding to CDK1 leading to G_2-M blockade. GADD45α also induces G1→S arrest by binding p21/Cip1; however, the underlying mechanism for G1→ S block has not been determined. Overexpression of GADD45α was found to inhibit cell proliferation (Zhan et al 1994). GADD45α is down-regulated in prostate cancer.

b) ***Differentiation***

VDR signaling can induce partial differentiation of prostate cancer cells since the gene expression signature of prostate cancer cells incubated with 1,25-D_3 was found to be similar in some respect to that specifying differentiated cells of the prostate epithelium (Guzey et al, 2004; Campbell et al, 1997; Chen & Holick, 2003; Deeb et al, 2007; Feldman et al, 2014). 1,25-D_3 treatment caused morphologic changes resembling induction of differentiation in the highly metastatic R3327-Mat-LyLu (MLL) Dunning rat

prostate cancer cells (Getzenberg et al, 1997). Consistent with findings by others, in unpublished study we observed that calcitriol treatment of LNCaP cells caused induction of androgen-inducible genes that are expressed in terminally differentiated prostate epithelial cells. Examples include the genes encoding the homeobox protein NKX3.1, prostate-expressed serine proteases of the kallikrein family (KLK2, KLK3 (*aka* PSA), KLK4, KLK15), TMPRSS2 and PMEPA1. We also observed calcitriol-mediated induction of *ID4* which encodes the inhibitor of DNA binding 4. ID4 is a dominant negative basic helix-loop-helix (bHLH) protein, which promotes normal prostate development and its level is markedly reduced in castration-resistant prostate cancer (Patel et al, 2014). Calcitriol-mediated induction of E-cadherin, an epithelial marker and cell-cell adhesion molecule, was observed in LNCaP and PC3 cells (Campbel, 1997). E-cadherin induction by calcitriol caused differentiation of colon cancer cells due to suppression of β-catenin activation (Palmer et al, 2001).

c) ***Apoptosis***

Several experimental systems demonstrated that 1,25-D_3-induced apoptosis can contribute to growth inhibition of prostate cancer cells (Blutt et al, 2000; Oades et al, 2002; Murthy et al, 2005; Saito et al, 2008; Washington & Weigel 2010). In one study, LNCaP and ALVA-31 cells, which underwent apoptosis upon calcitriol treatment, showed reduced expression of a number of anti-apoptotic proteins -- Bcl-2, Bcl-XL, MCI-1, BAG1L, XIAP, cIAP1 and cIAP2; the pro-apoptotic proteins BAX and BAK did not show altered levels. Bcl-2 overexpression prevented calcitriol-induced apoptosis of these cells. (Guzey et al, 2002). Caspase 3 and caspase 9, respectively the executor and initiator proteases in the mitochondria-dependent pathway to apoptosis, were induced by calcitriol, whereas caspase 8, which acts via the mitochondria-independent apoptotic pathway was not induced.

Another mechanism for calcitriol-induced apoptosis of prostate cancer cells involves a role for the insulin-like growth factor binding protein-3 (IGFBP3), which is induced by calcitriol due to VDR-

mediated transcriptional regulation of the IGFBP3 promoter (Boyle et al, 2001; Kojima et al, 2006). Calcitriol-mediated growth inhibition of LNCaP cells in serum-free media was found to depend on the induction of IGFBP3, which led to elevated expression of p21/Cip (Boyle, Feldman, 2001). Knockdown of IGFBP3 by RNA interference abrogated growth inhibition and p21 accumulation in these cells. However, calcitriol-regulated growth inhibition of LNCaP cells in serum-containing media did not require IGFBP3 induction (Stewart & Weigel, 2005). IGFBP3 overexpression in doxycycline-inducible cells induced apoptosis of LNCaP cells but not in C4-2 cells, although calcitriol can induce IGFBP3 in both LNCaP and C4-2 cells (Kojima et al, 2006). Thus, under certain experimental conditions the pro-apoptotic activity of IGBP3 caused growth inhibition of prostate cancer cells by vitamin D/VDR action.

The prostaglandin pathway and ERK/MAP kinase pathway, which promote cell survival, are additional mechanisms that lead to prostate cancer inhibition by vitamin D (Krishnan et al, 2007).

d) ***Angiogenesis, cell migration, metastasis***

Invasive tumor growth and metastasis requires angiogenesis, i.e. formation of new blood vessels from existing vessels – a process that is essential for primary tumor growth, progression to locally invasive carcinoma and then to metastasis at distant sites. Vitamin D signaling is thought to be active in both endothelial cells and smooth muscle cells of the vasculature since both cell types express VDR. 1,25-D_3 inhibited the angiogenic activity of endothelial cells. In a co-culture experiment, interleukin-8 (IL-8), secreted from PC3 and DU145 cells, would normally stimulate endothelial cell migration and tube formation, which are the two critical steps in angiogenesis. Calcitriol inhibited these processes by reducing *IL-8* expression – a consequence of inhibition of NF-κB activity by calcitriol (Bao et al, 2006). A role for 1,25-D_3 in inhibiting the angiogenic activity of tumor-derived endothelial cells *in vivo* was demonstrated in an allograft prostate tumor model (Chung et al, 2009). In this study, allograft tumors were produced in VDR-intact and VDR-null mice from prostate cancer cells isolated from tumors of TRAMP (Transgenic Adenocarcinoma of Mouse Prostate) mice. Tumors were much larger in VDR-

ablated mice at 30-day post inoculation of tumor cells. Furthermore, VDR signaling was detected for tumor-derived endothelial cells (TDECs) from wild type but not VDR-null mice, and calcitriol inhibited TDECs originating from wild type, VDR-expressing mice, not from VDR-null mice. Inhibition of angiogenesis by calcitriol was further supported by the findings that tumors from VDR-ablated mice had i) larger vascular volume; ii) enlarged vessels; iii) less pericyte coverage; iv) more vascular leakage; and v) higher levels of hypoxia-inducible factor1α (HIF1α), vascular endothelial growth factor (VEGF), angiopoietin 1 and platelet derived growth factor (PDGF-BB) (Chung et al, 2009). In a second example, when calcitriol or a potent analog (inecalcitol) was administered to mice bearing LNCaP xenograft tumors, reduction of the tumor burden was accompanied by decreased vascularity surrounding the tumor mass and within the tumor tissue (Okamoto et al, 2012).

1,25-D_3 inhibited cell motility and cell invasiveness. Calcitriol reduced the highly invasive phenotype of DU145 cells by suppressing expression of MMP9 and cathepsins, the proteases which promote motility and invasion (Bao et al, 2006). Metastasis of prostate cancer was inhibited by 1,25-D_3 in the syngeneic Dunning prostate tumor model, where tumors were subcutaneously produced in Copenhagen rats from highly aggressive MLL Dunning rat prostate cancer cells. 1,25-D_3 inhibited the growth of MLL tumors and reduced the number and size of lung metastases (Getzenberg et al, 1997).

4.2. Preclinical studies

A large number of preclinical studies involving prostate tumor xenografts and allografts and prostate cancer in genetically modified mice provided convincing evidence for the efficacy of calcitriol in reducing tumor burden and cancer metastases for androgen-dependent and castration-resistant prostate cancer (Deeb et al, 2007; Feldman et al, 2014). Furthermore, calcitriol acted synergistically with chemotherapeutics (Taxanes, platimum analogs) to reduce prostate tumor burden when administered either as a pretreatment or concurrently as a combined regimen (Hershberger et al, 2001). Several

synthetic vitamin D analogs (such as EB1089, Inecalcitol) have proven more effective as anti-cancer agents than the natural hormone (calcitriol) in studies with animal models (Perez-Stable et al, 2002; Okamoto et al, 2012). Dietary vitamin D_3 supplementation raised blood levels of 25(OH)D_3 and reduced tumor burden from xenografts of PC3 prostate cancer cells in athymic male nude mice (Swami et al, 2012). Interestingly, vitamin D_3 was as effective as calcitriol, which was injected intraperitoneally, in reducing tumor burden. In xenograft tumor tissue, dietary D_3 and injected calcitriol elevated, to similar levels, the mRNAs for CYP24A1, 15-PGDH, IGFBP-3 and p21/Cip, and also reduced mRNAs for cycloxygenase-2 and the prostaglandin receptor EP4 to similar levels. The mRNAs for Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) did not change. These results highlight the potential benefit of a combined regimen of nonsteroidal anti-inflammatory drugs (NSAIDs) and calcitriol/calcitriol analog or dietary vitamin D_3 for the clinical management of prostate cancer, as proposed (Krishnan et al, 2007; Swami et al, 2012).

It was recently reported that for prostate tumors in TRAMP mice, early intervention with calcitriol elevated the tumor tissue E-cadherin level (a pro-differentiation marker) and inhibited the androgen-stimulated growth of primary tumors during the early phase of the disease; however, continued calcitriol treatment increased distant organ metastases (Ajibade et al, 2014). TRAMP mice develop aggressive, metastatic prostate cancer in a stepwise manner – advancing from precancerous lesions to low-grade neoplasia and then to highly invasive carcinoma leading to distant metastases. The results of Ajibade et al indicate that long-term treatment of calcitriol may promote an aggressive disease phenotype. Whether increased miR-106-b levels and hence reduced expression of p21 (a miR-106-b target) would play a role for promoting disease progression in TRAMP mice under long-term calcitriol treatment should be assessed, since vitamin D can increase the microRNA miR-106-b level due to VDR-mediated induction of the DNA helicase *MCM7* gene (which harbors the miR-106 gene cluster within intron 13), and miR-106b targets p21/Cip for translational suppression (Thorne et al, 2011). In another study, a

chemoprevention role for calcitriol has been implicated based on the finding that in *Nkx3.1*; *Pten* mutant mice, calcitriol prevented PIN (prostate intraepithelial neoplasia) lesions from progressing to cancerous lesions (Banach-Petrosky et al, 2007).

4.3. Tumor-expressed VDR, association with lethal cancer, clinical potential of vitamin D

Malignant prostate tissue expresses VDR and supports VDR signaling, indicated by the tumor tissue expression of VDR target genes such as CYP24A1, IGFBP-3, COX-2, CYP3A4. Based on the data from 841 prostate cancer patients, it was concluded that high tumor tissue VDR expression is significantly associated with reduced risks for lethal prostate cancer (Hendrickson et al, 2011). Since the highest quartile of VDR expression was associated with reduced risks for the lethal disease, it was suggested that a threshold level of VDR may be needed in tumor tissue before VDR activity can have an impact on prostate tumor biology. In this patient cohort, VDR expression levels in cancer tissues did not associate with pre-diagnostic levels of serum 25(OH)D_3 and 1,25-D_3 and with VDR polymorphism.

In another study, however, VDR *FokI* polymorphism was found to associate with total prostate cancer risk (Li et al, 2007). *FokI* polymorphism produces the *F* allele which encodes a 3-amino acid-shorter, but functionally more active VDR variant compared to the longer VDR arising from the *f* allele. Translation initiation from the second ATG codon of the VDR mRNA generates the shorter VDR encoded by the *F* allele. Men with a low serum vitamin D status and a VDR genotype corresponding to the less active *f* allele are at ~2-fold higher risk for prostate cancer than men who carry the *FF* or *Ff* allele and have high serum 5(OH)D_3 plus 1,25$(OH)_2D_3$ (Li et al, 2007). Other VDR polymorphisms, i.e. *BsmI*, *ApaI*, *TaqI* polymorphisms –all located at the 3' end of the VDR gene outside of the coding region, and a poly A repeat did not show association with prostate cancer risk. These association studies indicate the possibility that the VDR genotype and expression, coupled with serum vitamin D status at initial diagnosis, may predict lethal versus indolent prostate cancer. Vitamin D and Omega-3 Trial (VITAL) is examining whether daily vitamin D supplementation at 2000 international unit (IU) with or

without ω-3 fatty acid supplementation (one gram fish oil daily) would reduce risks for prostate cancer and several other cancers, as well as risks for maladies like stroke and cardiovascular disease in healthy elderly men and women. ~20,000 participants are planned for the VITAL study; data gathering is scheduled to be completed by summer 2016 (Manson et al, 2012).

Despite extensive validation of the anti-proliferative and anti-tumor action of 1,25-D_3 in cell culture and animal studies, many clinical trials found no therapeutic benefit of calcitriol or its synthetic analogs as a single-agent anti-prostate cancer therapeutic, except for limited cases when partial tumor growth response and PSA response was observed (Feldman et al, 2014). A major clinical concern with vitamin D therapy is hypercalcemia, which develops in patients receiving a supra-physiological vitamin D dose necessary for tumor growth inhibition. In randomized controlled trials designed to assess the efficacy of a combined regimen of α-calcidol and docetaxel in patients with mCRPC, no benefit was observed for overall survival or PSA response (Attia et al, 2008).

Nevertheless, results from two recent small-scale studies are generating renewed optimism for vitamin D's role in prostate cancer chemoprevention, especially for the low-grade disease (Marshall et al, 2012; Hollis, 2015). A pilot study evaluated the effect of enhancing serum vitamin D status by vitamin D_3 supplementation (4000 IU per day) on prostate cancer progression for 44 patients diagnosed with low-risk prostate cancer who chose active surveillance over radical prostatectomy. More than 50% of patients who took daily vitamin D_3 supplement for one year had decreased number of positive cores at repeat biopsy, decreased Gleason scores and disappearance of some tumors (Marshall et al, 2012). 34 % showed increase in the number of positive cores and increased Gleason scores. 11% showed no change in tumor pathology. Serum PSA levels did not change. No adverse health consequences were observed due to year-long vitamin D_3 supplementation. In another pilot study, 37 men awaiting elective prostatectomy were randomized into two groups – one received vitamin D_3 daily (4000 IU) for two

months and the other group received placebo (Hollis, 2015). After two months, analysis of resected prostate tissues showed that for 60% in the vitamin D group, tumors shrank or disappeared and the tissue had dramatically reduced inflammation, judged from decreased levels of inflammation-related lipids and proteins and induction of the growth factor differentiation factor-15 (GDF-15). GDF-15, which counteracts inflammation, is markedly reduced in prostate cancer (Hollis, 2015). The placebo group showed either no change in tumor or tumor progressed. These results are consistent with the anti-inflammatory role of vitamin D (Krishnan & Feldman, 2011), and it should be noted that inflammation is strongly linked to various cancers including prostate cancer (DeMarzo et al, 2007). Since preclinical studies showed that 1,25-D_3 reduced the tumor tissue prostaglandin level by reducing expression of COX-2 and the prostaglandin receptor and inducing 15-prostaglandin dehydrogenase, it is important to know whether combining vitamin D_3 supplementation with an NSAID, as proposed by Feldman and colleagues (Moreno et al, 2006), would inhibit slow-growing as well as aggressive prostate cancer.

5. FUNCTIONAL INTERPLAY OF AR AND VDR IN PROSTATE CANCER: IMPACT ON CELL GROWTH AND INTRACRINE ANDROGEN BIOSYNTHESIS

5.1. Impact on Cell Growth:

Crosstalk between VDR and AR signaling in prostate cancer cells was demonstrated in cell culture and animal model studies. Androgen-induced AR signaling was found to contribute to the 1,25-D_3-mediated inhibition of androgen-dependent (LNCaP, CWR22R) and castration-resistant (C4-2) prostate cancer cells, since inhibition of these AR-positive cells was blocked by Casodex (an AR antagonist) or by AR silencing (via RNA interference) or by targeted disruption of genomic AR (Zhao et al, 2000; Bao et al, 2004; Lau et al, 2004; Murthy et al, 2005). In calcitriol-treated LNCaP cells, androgen-induced hyperphosphorylation of pRb was prevented, despite elevation of the AR protein level (Lau et al, 2010). However, an androgen-independent mechanism can also contribute to the inhibition, since Casodex did not prevent calcitriol-induced inhibition of other prostate cancer lines such as the MDA lines (PCa-2a,

PCa-2b); LAPC4; CWR22RV1; ALVA-31 and LNCaP-104R1, even though functional AR is present in all of these cell lines (Zhao et al, 2000; Yang et al, 2002;Weigel, 2007).

Crosstalk of VDR with AR pathways *in vivo* is indicated by the finding that testosterone supplementation abolished VDR-dependent differential tumor growth and tumor cell proliferation in the LPB-Tag (long probasin promoter-driven SV40 T antigen) model of mouse prostate cancer (Mordan-McCombs et al, 2010). In this study, significantly higher tumor growth and tumor cell proliferation were observed in LPb-Tag x VDR-KO mice compared to LPb-Tag x VDR-intact mice, whereas testosterone administration obliterated these differences between the two groups. In epidemiologic research, inter-individual differences in serum testosterone levels may have contributed to inconsistent results on the relationship between prostate cancer risk and serum vitamin D status (Mordan-McCombs et al, 2010). Further evidence for an interacting role of androgen and vitamin D signaling in the regulation of prostate growth comes from the finding that 1α, 25-D_3 reduced the prostate size of testis-intact but not castrated Sprague Dawley rats (Leman et al, 2003). Finally, tumor growth rate, enhanced by factors derived from endothelial cells within the prostate cancer microenvironment, may be modulated due to VDR-AR interaction, since serum testosterone and 25(OH)D_3 levels markedly influenced blood flow and vascularity of rat prostate, and both of these nuclear receptors are expressed in the endothelial cells of normal and malignant prostate (Godoy et al, 2013).

5.2. Calcitriol, androgen and intracrine androgen metabolism in prostate

Metabolic inactivation of AR ligands can potentially be a fruitful approach for targeting advanced prostate cancer, since androgen-induced AR activity is a key driver of prostate cancer progression. Increased intracrine androgen biosynthesis in cancer tissue from the adrenal dehydroepiandrosterone (DHEA) is an important mechanism for activating the AR axis in castration-resistant prostate cancer (Mostaghel & Nelson, 2008). Indeed, the androgen biosynthesis blocker Zytiga® (abiraterone acetate)

can reduce tumor tissue AR activity and the drug is widely used in clinical practice to control castration-resistant prostate cancer (Mostaghel, 2014). It was reported that calcitriol can induce the cytochrome P450 enzymes in androgen-dependent LNCaP and LAPC4 prostate cancer cells, and CYP3A4 induction in LNCaP cells was shown to induce oxidative inactivation of testosterone (to 6β-hydroxy-testosterone) and DHEA (to 16α-hyroxy-DHEA) (Maguire et 2012; Doherty et al, 2014). Reduced CYP3A4 expression in specimens from aggressive prostate cancer and in circulating prostate cancer cells has been reported (Fujimura et al, 2009; Mitsiades et al, 2012).

Calcitriol and the analog EB1089 caused a 2- to 3-fold induction of SULT2B1b sulfotransferase mRNA and protein in prostate cancer cells and *in vivo* in mouse prostate, and a DR7-type VDRE is involved in the VDR-mediated induction of the corresponding gene (*SULT2B1*, Seo et al, 2013). The C-3 hydroxyl group of DHEA and cholesterol is targeted by SULT2B1b (aka SULT2B) for sulfoconjugation, which facilitates their metabolic clearance. Silencing of SULT2B by RNA interference caused accelerated proliferation of prostate cancer cells in culture (Seo et al, 2013) and in xenograft tumors (Park et al, in preparation). Abundant SULT2B expression in normal prostate epithelium is contrasted by its reduced level in primary prostate cancer and its almost negligible expression in metastatic CRPC (Fig.3). No detectable SULT2B was observed in >90% cases of distant metastases that we have analyzed. Occasionally, weak SULT2B staining in metastatic specimens was detected -- a representative example being the specimen at lower panel, right (Fig. 3).

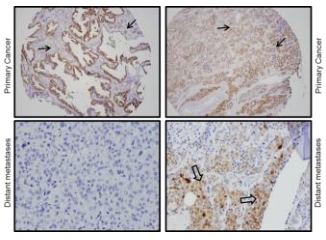

Figure 3. SULT2B-specific immunostaining of prostate cancer specimens from four cases. **Upper panel**: Primary prostate cancer from two patients; photomicrographs taken at 4X. **Lower panel**: distant metastases of prostate cancer from two patients; photomicrographs taken at 20X.

The primary specimen at left shows non-malignant acini, which stained strongly for SULT2B, as well as cancerous areas (solid arrows), which show markedly reduced SULT2B levels. The tissue core at the right shows only the cancer region (open arrows), which stained weakly for SULT2B. (Specimens were generously provided by Dr. Elahe Mostaghel, MD, PhD and Colm Morrissey (Univ Washington, Seattle).

The above findings make it likely that the prostatic androgen flux is regulated in part by SULT2B activity, and interference with this flux due to reduced SULT2B expression, as observed in primary

cancer and in mCRPC, would promote prostate cancer growth by enhancing tumor tissue androgen synthesis. Confirmation of this possibility awaits experimental evidence.

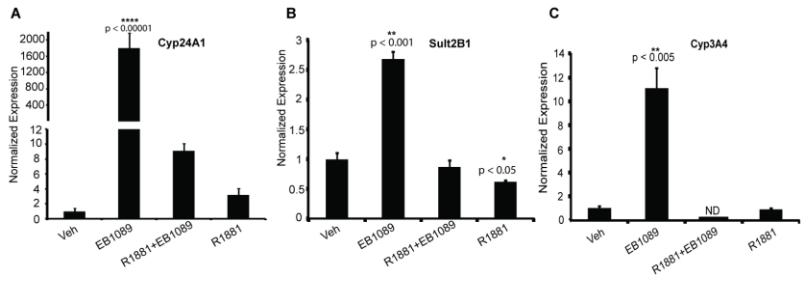

Figure 4. QRT-PCR assay of mRNAs for CYP24A1 (A), SULT2B1 (B), CYP3A4 (C) in C4-2B human castration-resistant prostate cancer cells treated with vehicle, EB1089 (10 nM), EB 1089 (10 nM) + R1881 (1 nM), or R1881 (1nM)

Treatment of C4-2B castration-resistant cells concurrently with R1881 (a synthetic androgen) and EB1089 completely abrogated the induction of SULT2B1 mRNAs observed with EB1089 alone (Fig. 4B). Similarly, EB1089 induced CYP3A4 mRNA expression by more than 10-fold, while co-treatment with R1881 drastically reduced the level of CYP3A4 mRNAs, bringing it to the basal level or even lower (Fig.4C). EB1089-induced robust expression of CYP24A1 (~2000-fold) was also drastically reduced (to less than 10-fold) by the combined action of R1881 and EB1089 (Fig.4A). Dampened vitamin D/VDR-induced CYP24A1 mRNA expression in the presence of androgen is beneficial, as it would prevent ligand degradation, thereby enhancing VDR signaling.

An important example of the beneficial effect of androgen-vitamin D interaction is the inhibition of AR-regulated energy metabolism of LNCaP prostate cancer cells (Wang et al, 2013; Wang & Tenniswood, 2014). Normally, prostate cancer cells generate energy using an aerobic mechanism that requires citrate oxidation by m-aconitase-2 activity and propagation of TCA cycle. Glucose usage stimulated by androgen-activated AR leads to the production of acetyl CoA, which feeds into the TCA cycle for citrate synthesis (Costello & Franklin, 1991a,1991b). Combined 1α,25-D_3 and testosterone treatment of LNCaP cells caused up-regulation of zinc transporters (SLC39A1 and SLC39A11) that led to intracellular zinc accumulation, inactivation of m-aconitase-2 activity at the high zinc level and secretion of citrate to the cytoplasm for lipid synthesis; also, reduced expression of the thiamine pyrophosphate (TPP) transporter (SLC25A19) in the presence of 1α,25-D_3 and testosterone together

would lower the mitochondrial level of the coenzyme TPP, which in turn can decrease the activities of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase and inhibit ATP production (Costello & Franklin, 1991a,b; Wang et al, 2011; Wang and Tenniswood, 2014). Our observation that androgen dramatically reduced vitamin D-induced expression of CYP24A1 mRNAs in castration-resistant C4-2B cells implies that the vitamin D level in these cells would remain at a relatively high level. This result seems to predict that androgen supplementation, at least intermittently, has the potential to enhance the anti-prostate tumor activity of calcitriol or analog. Human trials are needed to assess the potential of combined vitamin D and testosterone supplementation in prostate cancer prevention and treatment.

6. SUMMARY AND FUTURE POSSIBILITIES

Besides its essential role in bone mineralization, the bioactive vitamin D (i.e. calcitriol, 1α, 25-D_3 or synthetic analogs) has wide impact on the physiology and pathophysiology of extraskeletal tissues, including growth inhibition of normal and malignant tissues of prostate. The efficacy of vitamin D for inhibiting prostate cancer cells and prostate tumor growth in cell culture and animal models is well established. However, clinical trials with calcitriol or its analog as a single agent are inconclusive, especially since the high dose of vitamin D needed for clinical efficacy is known to induce hypercalcemia. Non-significant association of a low serum vitamin D status with increased prostate cancer risk further adds to the uncertainty for the beneficial role of vitamin D as a single agent in prostate cancer therapy and chemoprevention. On the other hand, encouraging results were observed in two recent small-scale pilot studies. In one study, progression of low-grade prostate cancer was prevented or reversed for patients on active surveillance who received daily, safe-dose vitamin D supplementation, at 4000 IU each day for a one-year period. In another study, after daily vitamin D supplementation (4000 IU/day) was given for two months to patients who elected surgery, cancer tissue in resected prostate samples showed markedly decreased inflammation, induction of the anti-inflammatory protein GDF-15 (which is reduced in prostate cancer) and lower Gleason score. These

results (discussed in section 4.3) indicate that vitamin D may inhibit prostate cancer progression in humans, at least for the low-grade, organ confined disease.

The genomic action of vitamin D is mediated by the cognate vitamin D receptor (VDR) which is a ligand-inducible, DNA-binding transcription factor and a member of the nuclear receptor superfamily. Non-genomic action of vitamin D through a membrane receptor plays a relatively minor role in the regulation of cell functions. VDR has a multi-domain organization similar to other nuclear receptors. Due to inter-domain allosteric interactions, the transcriptional activity of VDR is influenced by ligand structure, nucleotide organization of DNA response elements in target genes. Calcitriol is biosynthesized in human body from 7-dehydrocholesterol, which is converted in sunlight-exposed skin to the secosteroid vitamin D_3 (abbreviated D_3), which is then sequentially converted to 25-hydroxy D_3 in the liver, predominantly by CYP2R1, and then to 1α, 25-dihydroxy D_3 (1, 25-D_3) by the 1α-hydroxylase activity of CYP27B1 in the kidneys. Local synthesis of 1, 25-D_3 in prostate and other extrarenal tissues from circulating 25-hydroxy D_3 is mediated by the 1α-hydroxylase activity of CYP27B1. Degradation of 1, 25-D_3 is mediated by the 24-hydroxylase activity of the catabolic enzyme CYP24A1. Vitamin D homeostasis under normal physiology is maintained by a regulatory loop that entails 1, 25-D_3-mediated induction of *CYP24A1* and repression of *CYP2R1* and *CYP27B1*. Overexpression of CYP24A1 in advanced prostate cancer has been reported (Tannour-Louet et al, 2014).

Functional interaction between the AR and VDR axis in prostate cancer cells has been demonstrated in experimental systems. This crosstalk may facilitate vitamin D action against prostate cancer. AR, which promotes disease progression in mCRPC, is activated by tumor tissue androgen, which is produced by intracrine androgen biosynthesis from the adrenal steroid DHEA. The prostate-expressed SULT2B1b (aka SULT2B), a phase II sulfotransferase, may interfere with intracrine androgen production since conversion of DHEA to DHEA-sulfate by SULT2B would lower the precursor DHEA

pool available for intracrine androgen synthesis. A role for SULT2B in prostate cancer progression is likely since its expression is significantly reduced in primary cancer and it is mostly non-detectable in cancer tissues of distant metastases. Vitamin D (calcitriol and EB1089) induced SULT2B expression in castration-resistant prostate cancer cells. The phase I enzyme CYP3A4, which inactivates DHEA and testosterone by oxidative modification, is induced by VDR in castration-resistant prostate cancer cells. Therefore, vitamin D may attenuate AR signaling in advanced prostate cancer by restricting the ligand source for AR. The observed induction of CYP24A1 by vitamin D in prostate cancer cells would antagonize vitamin D action, especially since CYP24A1 overexpression in advanced prostate cancer has been reported. We found that androgen suppressed VDR-mediated CYP24A1 induction in prostate cancer cells. Thus, VDR action may lower the ligand pool for AR (due to induction of CYP3A4, SULT2B) and AR activity may help protect the VDR ligand from CYP24A1-mediated degradation. However, VDR-mediated induction of SULT2B and CYP3A4 was also suppressed by androgen, which can potentially elevate AR signaling by preventing reduction of the tumor tissue androgen level. Elevated AR signaling, on the other hand, may further suppress VDR-induced CYP24A1 expression and provide additional protection against 1,25-D_3 degradation. Studies in animal models may reveal the net impact of androgen supplementation (continuously or intermittently) on the inhibition of prostate tumor growth by bioactive vitamin D.

Design of future clinical trials of vitamin D should take into consideration a number of results from *in vitro* and *in vivo* studies. For example, calcitriol induced the microRNA miR106-b as well as the cell cycle inhibitor p21 in prostate cancer cells (discussed in section 4.1). Since p21 is a target of miR106-b, under certain conditions, calcitriol-induced expression of miR106-b may prevent accumulation of p21 so that vitamin D may fail to cause inhibition of prostate tumor growth. In human trials, the combined action of calcitriol (or analog) and an oligonucleotide-based anti-miR, which targets miR106-b and specifically reduces its endogenous level, may be investigated. In another example, calcitriol inhibited

the prostaglandin pathway by down-regulating the expression of COX-2 and prostaglandin receptor and up-regulating 15-prostaglandin dehydrogenase (discussed in section 4.2). Therefore, calcitriol combined with a non-steroidal anti-inflammatory drug (NSAID) may be a potent regimen for inhibition of prostate cancer progression (Moreno et al, 2005). Vitamin D can induce growth arrest of prostate progenitor/stem cells (Li et al 2009; Maund et al, 2011) and the natural compound sulfarophane, an organosulfur present in cruciferous vegetables, was shown to ablate pancreatic cancer stem cells (Hu & Fu, 2012). It will be of interest to assess the effect of the combined action of vitamin D and sulfarophane on prostate tumor growth in humans. Finally, a number of low calcemic alternatives to calcitriol are now available. Inecalcitol is one such example; it has higher anti-proliferative activity and is 100-fold less hypercalcemic than calcitriol. Inecalcitol was reported to be highly effective in inhibiting the growth of LNCaP cells in culture and in xenograft tumors (Okamoto et al, 2012), and a Phase I multi-center trial of inecalcitol combined with docetaxel has shown encouraging PSA response in naïve mCRPC patients (Medioni et al, 2014). With advances in the development of next-generation calcitriol analogs and recent encouraging pilot data showing efficacy of calcitriol in inhibiting low-grade prostate cancer at a safe, physiologically compatible dose, it is likely that calcitriol or synthetic analogs will be approved in the near future for use in prostate cancer prevention and treatment.

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Figure Legend

Figure 1. Schema showing enzyme catalyzed pathways to the biosynthesis and degradation of calcitriol. Regulation of CYP2R1 and CYP27B1 by calcitriol (1α,25-dihydroxy vitamin D_3), parathyroid hormone (PTH), phosphorous (Pi), calcium (Ca^{2+}) and fibroblast growth factor-23 (FGF23) is shown. Stimulation is indicated by a solid arrow (⟶); inhibition is shown by a broken arrow (- - - ⇢).

Figure 2. **(A)** Electrophoretic mobility shift assay (EMSA) showing binding of VDR and RXR-α (present in the nuclear extract of normal human prostate tissue) to the DR7-type element present in around the -202 nucleotide position of the *SULT2B1* promoter. The promoter sequence containing the DR7-type element is shown. **Left panel**: oligonucleotide competition demonstrates specificity of the EMSA complex; antibody supershift assay demonstrates the presence of VDR and RXR-α within the EMSA complex. **Right panel**: Relative affinities of DR7 and a DR3-type VDRE (from the rat osteocalcin promoter) for the EMSA complex formed by ^{32}P-labeled DR7 element with VDR and RXR-α components of human prostate nuclear extract. These data from our experiments are taken from Seo et al, *Mol Endocrinol*, 2013. **(B)** Subcellular localization of transfected CFP-VDR in COS1 cells treated with ethanol or 10 nM calcitriol. Representative data from two separate transfection experiments are shown. CFP= cyan fluorescent protein.

Figure 3: Photomicrographs of SULT2B-immunostained prostate cancer specimens from four cases. **Upper panel**: Primary prostate cancer from two patients; photomicrograph taken at 4X. The primary specimen at left shows non-malignant acini, which stained strongly for SULT2B, as well as cancerous

areas (solid arrows), which show markedly reduced SULT2B levels. The tissue core at the right shows only the malignant region of primary cancer (arrows), which stained weakly for SULT2B. **Lower panel**: distant metastases of prostate cancer from two patients; photomicrograph taken at 20X.

Figure 4: CYP24A1, SULT2B1 and CYP3A4 mRNAs in C4-2B castration-resistant human prostate cancer cells treated with vehicle; EB1089 (10 nM); R1881 (1 nM) + EB1089 (10 nM); R1881 (1 nM). Panel A: CYP24A1 mRNAs. Panel B: SULT2B 1b mRNAs. Panel C: CYP3A4 mRNAs. Cells were incubated with indicated hormones or vehicle for 20hrs before RNAs were extracted from the treated cells and analyzed by qRT-PCR assay

REVISED VERSION

Nuclear Receptors in Drug Metabolism, Drug Response and Drug Interactions

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Abbreviations NR, nuclear receptor; DBD, DNA-binding domain; LBD, ligand-binding domain; XRE, xenobiotic response element, PXR, pregnane X receptor; CAR, constitutive androstane receptor; VDR, vitamin D receptor; FXR, farnesoid X receptor; LXR, liver X receptor; CYP, cytochrome P450; DME, drug-metabolizing enzyme; ADME, absorption, distribution, metabolism, excretion; DDI, drug-drug interaction; PTM, post-translational modification; MDR, multi-drug resistance; ABC, ATP-binding cassette; HDAC, histone deacetylase; HAT, histone acetyltransferase; HMT, histone methyltansferase; HMD, histone demethylase; DNMT, DNA methyltransferase; SNP, single nucleotide polymorphism

ABSTRACT

Orally delivered small-molecule therapeutics are metabolized in the liver and intestine by phase I and phase II drug-metabolizing enzymes (DMEs), and transport proteins coordinate drug influx (phase 0) and drug/drug-metabolite efflux (phase III). Genes involved in drug metabolism and disposition are induced by xenobiotic-activated nuclear receptors (NRs), i.e. PXR (pregnane X receptor) and CAR (constitutive androstane receptor), and by the 1α, 25-dihydroxy vitamin D_3-activated vitamin D receptor (VDR), due to transactivation of xenobiotic-response elements (XREs) present in phase 0-III genes. Additional NRs, like HNF4-α, FXR, LXR-α play important roles in drug metabolism in certain settings, such as in relation to cholesterol and bile acid metabolism. The phase I enzymes CYP3A4/A5, CYP2D6, CYP2B6, CYP2C9, CYP2C19, CYP1A2, CYP2C8, CYP2A6, CYP2J2, and CYP2E1 metabolize >90% of all prescription drugs, and phase II conjugation of hydrophilic functional groups (with/without phase I modification) facilitates drug clearance. The conjugation step is mediated by broad-specificity transferases like UGTs, SULTs, GSTs. This review delves into our current understanding of PXR/CAR/VDR-mediated regulation of DME and transporter expression, as well as effects of single nucleotide polymorphism (SNP) and epigenome (specified by promoter methylation, histone modification, microRNAs, long non coding RNAs) on the expression of PXR/CAR/VDR and phase 0-III mediators, and their impacts on variable drug response. Therapeutic agents that target epigenetic regulation and the molecular basis and consequences (overdosing, underdosing, or beneficial outcome) of drug-drug/drug-food/drug-herb interactions are also discussed. Precision medicine requires understanding of a drug's impact on DME and transporter activity and their NR-regulated expression in order to achieve optimal drug efficacy without adverse drug reactions. In future drug screening, new tools such as humanized mouse models and microfluidic organs-on-chips, which mimic the physiology of a multicellular environment, will likely replace the current cell-based workflow.

INTRODUCTION

Drug metabolism, which occurs primarily in the liver and intestine, refers to the enzymatic modification and subsequent disposal of medicinally active compounds, originating either endogenously (as steroids, neurotransmitters, metabolic products like bile acids) or exogenously (as natural products or synthetic/semi-synthetic chemicals). Upon conversion to hydrophilic metabolites, drugs are eliminated from the body following biliary excretion and renal clearance by glomerular filtration and tubular secretion. Drug metabolism is also integral to the biotransformation of pro-drugs to pharmaco-active agents. Drug metabolism and disposition is coordinated by an array of liver- and intestine-expressed drug-metabolizing enzymes (DMEs) and drug-transporting proteins whose tissue abundance is transcriptionally regulated by specific nuclear receptors (NRs), which are ligand-activated transcription factors (*Willson & Kleiwer, 2002*).

Of the 48 distinct receptors comprising the NR superfamily in humans, pregnane X receptor (PXR, NR1I2) and constitutive androstane receptor (CAR, NR1I3) are primary transcriptional regulators of the genes involved in the metabolism and elimination of drugs/drug metabolites (*Xie & Evans, 2001*; *Tzameli & Moore, 2001*; *Evans & Mangelsdorf, 2014*). PXR and CAR are generically referred as xenobiotic NRs, since they are activated by structurally diverse drugs and environmental xenobiotics. PXR and CAR are also activated by a number of endobiotics (steroids, sterols, retinoids, thyroid hormones, bile acids). In addition, PXR and CAR activation can result from receptor phosphorylation by various kinases that are activated in response to drug-mediated induction of specific intracellular signal cascades; in this case, drugs may not directly interact with the xenobiotic NRs (*Smutny et al, 2013*). Vitamin D receptor (VDR, NR1I1), beyond its classic role in calcium and phosphate homeostasis, has the ability to transcriptionally induce drug transporters and DMEs, especially in the enterocytes of intestinal tissue (*Makishima et al, 2002*; *Chatterjee et al, 2005*). In certain contexts,

additional NRs, such as the bile acid-activated farnesoid X receptor (FXR, NR1H4); oxysterol-activated liver X receptor (LXR-α, NR1H3); fatty acid/eicosanoid-activated peroxisome proliferator activated receptor (PPAR-α, NR1C1), and retinoid-related orphan receptors (ROR-α, ROR-γ) regulate genes linked to drug absorption, distribution, metabolism and excretion (ADME) (*Xie & Chiang, 2013*). Hepatocyte nuclear factor (HNF4-α, NR2A1), a member of the NR superfamily, plays a synergizing role in the PXR- and CAR-mediated transactivation of DME- and transporter-encoding genes (*Tirona et al, 2003*; *Echchgadda et al, 2007*; *Hwang-Verslues & Sladek, 2010*).

Altered activities of polymorphic variants of NRs and ADME-related gene products account for variable response to prescription medicine between individuals. Amino acid changes due to nucleotide polymorphisms in the coding region can influence protein stability or activity, while polymorphism at upstream, downstream or intragenic regulatory loci can alter NR-mediated ADME gene transactivation. Epigenetic, transcriptional and posttranslational regulation of xenobiotic NRs can further impact drug metabolism and clearance. Evaluation of drug-drug interactions (DDI), which result from changes in the level or activity of DMEs and/or transporters due to a second drug, is an essential component of drug development workflow.

In this review, we describe various classes of DMEs and transporters, present an overview of the molecular underpinnings for NR-mediated genetic and epigenetic regulation of ADME genes and consider roles for various NRs (especially PXR/CAR/VDR) and their target genes in differential drug response. Illustrative examples highlighting critical roles of xenosensing NRs, DMEs and transporters in DDI are also examined. Finally, we discuss current drug-screening platforms and their potential future improvements.

1. Drug metabolizing enzymes (DMEs) and drug transporters: The four phases of drug metabolism entail cellular uptake of therapeutic molecules (phase 0); their enzymatic oxidation (phase I) and conjugation (phase II), and efflux of drug metabolites for clearance (phase III). PXR and CAR activate genes involved in all four phases. General steps in drug metabolism and elimination are shown in **Fig. 1**.

1.a *Phase 0 uptake proteins*:

Basolaterally located uptake proteins guide cellular entry of drugs from circulation; drug influx can be a rate-limiting step for drug metabolism and clearance (*König et al, 2013; Döring and Petzinger, 2014*). All uptake proteins are members of the solute carrier (SLC) protein family of which there are more than 300 members grouped under 52 subfamilies. Liver, intestine and kidneys are major sites of SLC expression. Most SLC proteins localize to cell membrane, although some may localize to mitochondria and other organelles. Proteins from nineteen *SLC* gene subfamilies have drug uptake activity. Most significant gene families of uptake transporters are *SLC15* (oligopeptide transporter), *SLC22* (organic anion/cation/zwitterion transporter), *SLCO* (organic anion transporting polypeptide) and *SLC47* (organic cation transporter) (*Russell, 2010; Nigam, 2015*). For example, OCT1 is a *SLC22A1* encoded organic cation uniporter involved in the influx of the antiviral agent acyclovir, ganciclovir and the anti-diabetic drug metformin. Drug substrates for proteins encoded by *SLC15, SLC22, SLCO* and *SLC47* families have been described (*Russel 2010; König et al, 2013; Nigam, 2015*). SLCs either serve as channels (uniporter) to guide drug diffusion down an electrochemical gradient, or drive drug transport against a diffusion gradient that is coupled to the symport or antiport of small inorganic or organic ions.

1.b *Phase I DMEs*

Heme-containing cytochrome P450s (CYPs), flavin-containing monooxygenases, monoamine oxidases and xanthine oxidase/aldehyde oxidases are examples of phase I DMEs, which generally

localize to the endoplasmic reticulum of cells. CYP enzymes play the most prominent role in phase I metabolism. Liver is the first pass and primary site of phase I metabolism, along with the gastrointestinal tract, kidneys, skin, and lung serving as additional sites; most tissues, however, express phase I DMEs. Addition or exposure of polar functional groups (e.g. -OH, hydroxyl; -COOH, carboxyl; -NH2, amine; -SH, sulfhydryl) to drug substrates enhances their bioavailability and solubility and promotes pro-drug biotransformation. Polar groups also arise by reduction of a ketone or aldehyde group to an alcohol; oxidation of an alcohol to an acidic group; hydrolysis of esters and amides; reduction of azo and nitro groups; oxidative dealkylation of N-alkyl, O-alkyl , and S-alkyl groups. When sufficiently polar, phase I metabolites can be pumped out of cells without additional modification.

CYPs are products of a multigene family, which for humans include 57 *CYP* genes (*Zanger & Schwab, 2013*). Individual CYP is specified by the family (with an Arabic numeral), then the subfamily (with a letter) followed by the isozyme within the subfamily (with another Arabic numeral) and the allele number (with a preceding asterisk) of an individual gene within the subfamily. As an example, for CYP2D6*1, the *1 allele is wild type CYP2D6 with normal activity. Additional alleles of CYP2D6, marked with higher numbers preceded by *, exhibit aberrant functions (*Wijnen et al, 2007*). CYPs are expressed in practically all tissues, with liver exhibiting the highest abundance and expressing largest number of individual CYPs. Enzymes of the CYP-1, -2 and-3 families metabolize majority of drugs and non-drug xenobiotics. The fraction of clinical drugs that are substrates for individual CYPs is schematically presented as **Figure 2.** CYP3A4, the most abundant CYP enzyme in human liver, acts on the greatest number of drugs and other xenobiotics. CYP2D6, although present at lower abundance, metabolizes numerous drugs. Substrate specificity is narrower for other members of the CYP family that are expressed in hepatic and extrahepatic tissues. They target endogenous substrates like sterols, fatty acids, eicosanoids and vitamins. A comprehensive list of drug substrates for CYPs has been

reported (Lynch & Price, 2007; *www. pharmacologyweekly.com/cytochrome-cyp-p450-enzyme-medication-herbs-substrates, updated May, 2015*).

1.c. *Phase II DMEs*

Broad-specificity phase II transferases catalyze conjugative reactions. Common phase II modifications are glucuronidation by UDP-glucuronyltransferase (UGT), sulfonation by sulfotransferase (SULT), glutathionylation by glutathione S-transferase (GST), acetylation by N-acetyl transferase (NAT) and methylation by methyltransferase (MT). For any given transferase family, individual family members show predilection for a distinct set of substrates. Cofactors of phase II transferases react with functional groups that are either part of the parent drug or generated by phase I modification. In contrast to the enhanced potency of many phase I metabolites, phase II modified drug metabolites normally exhibit diminished function. PXR- and CAR-mediated gene regulation for a number of phase II transferases has been studied (*Echchgadda et al, 2007*; *Tolson & Wang, 2010*).

1.d. *Phase III Efflux Proteins*

Members of the ATP binding cassette (ABC) superfamily, encoded by the *ABCB*, *ABCC*, and *ABCG* gene subfamilies, are broad-specificity exporters that pump drugs out of cells using energy from ATP hydrolysis. In hepatocytes, efflux proteins reside either in canalicular/apical membranes or in blood-facing basolateral/sinusoidal membranes, guiding drugs, endobiotics and their metabolites for biliary excretion and efflux into systemic circulation. Multidrug-resistance associated proteins MRP2 (ABCC2), the bile salt export pump BSEP (ABCB11), the breast cancer resistance protein BCRP (ABCG2) are examples of ABC cassette family transporters which mediate apical efflux of drugs, steroids, bile acids and their conjugates. P-glycoprotein (MDR1, ABCB1) is an apical membrane transporter in hepatocytes (*Nigam, 2015*). Basolateral efflux of unconjugated and phase II-conjugated

drugs, steroids, prostaglandin and bile acids from hepatocytes into sinusoidal blood is assisted by ABC transporters such as the multi-drug resistance associated proteins MRP3, MRP4, MRP5 and, also, by the ATP-independent OSTα/OSTβ complex that functions as an organic solute and steroid transporter. OSTα contains seven transmembrane domains and OSTβ has a single transmembrane domain (*Ballatori, 2005*), and neither are part of the ABC transporter superfamily. MATE (multidrug and toxin extrusion) efflux transporters are H+-coupled antiporters, which transport structurally unrelated organic cations out of cells. They are members of the solute carrier subfamily SLC47, expressed primarily in the liver and kidney, and they localize at apical membranes of renal tubular epithelia and bile canaliculi. MATE1 (product of *SLC47A1*) mediates organic cations extrusion into urine and bile. In the human kidney, the uptake transporter OCT2 (organic cation transporter, *SLC22A2* encoded) promotes the import of cationic drugs (such as metformin, cisplatin, imatinib) from the blood at the basolateral membrane of the proximal tubule epithelial cells. MATE-1 and the isoform MATE-2K mediate secretion of cationic drugs across the brush-border membrane into the proximal tubule lumen *(Motohashi & Inui, 2013).*

2. Nuclear receptors (NRs), response elements, gene regulation by PXR/ CAR/VDR:

NRs, upon association with DNA response elements, induce a cascade of protein-protein interactions that lead to the assembly of multiple classes of regulatory proteins (coactivators, corepressors, histone modifiers, chromatin remodeling complex) at the NR-bound chromatin region. Signal transmission from the coregulator assembly to the basal transcription machinery via a multi-protein mediator complex culminates in altered RNA polymerase II activity and transcriptional response of NR-regulated genes.

The NR superfamily of ligand-activated transcription factors in humans is defined by 48 receptors grouped into four classes (Type I-IV) based on the nature of activating ligands, preferred sequence organization of NR-binding DNA response elements in target genes and dimerization partner of the activated NR (*Mangelsdorf et al, 1995; Sever & Glass, 2013*). Type I NRs reside in the cytoplasm in an inactive state in association with chaperone proteins. They are activated upon binding cognate steroid hormone ligands, translocated to the nuclear compartment and bind target gene response elements as homodimers to mediate gene regulation. Type II receptors, such as the vitamin D receptor (VDR), are activated by non-steroid endocrine ligands (1α,25-dihydroxy vitamin D_3 (1,25-D_3, in short) for VDR; retinoic acid-all trans, for RAR-α/-β/-γ; thyroid hormone for TR-α/-β). Several Type II receptors are activated by intracrine ligands (e.g. bile acids activating FXR-α; oxysterols activating LXR-α/-β; fatty acids/eicosanoids activating PPAR-α/-γ/-δ). Type II NRs in an inactive state remain tethered to corepressors as heterodimers with the obligate partner retinoid X receptor (RXR). Exchange of corepressors for coactivators initiates activation of ligand-bound Type II NRs. PXR and CAR, comprising the Type III subgroup, are transported from cytoplasm to the nucleus upon activation by chemical inducers. Nuclear PXR and CAR bind to DNA response elements as dimers with RXR to set the stage for subsequent regulation of target gene transcription. Activation of CAR in most cases entails a ligand-binding independent mechanism, as reported for phenobarbital-like chemicals, which induce dephosphorylation of CAR at threonine-38, thereby activating CAR and promoting its nuclear translocation. Direct ligand binding and activation of CAR has also been reported for some xenobiotic compounds (*Mutoh et al, 2013*; *Yang & Wang, 2014*). Type IV NRs (e.g. LRH1, NGF1-B/NUR77, RORs) bind to DNA elements as a monomer, homodimer, or even as a heterodimer, partnering with RXR or another member of the same subfamily (*Mullican et al, 2013*). Although PXR, CAR and, to a significant extent VDR, are primary regulators of drug metabolism and disposition, NRs from all four classes are known to influence drug/xenobiotic response, as discussed under section 2c.

For all NRs, the primary structure specifies a common generalized organization based on functional domains (*Sever & Glass, 2013*; *Evans & Mangelsdorf, 2014*). The highly variable amino-terminal A/B domain harbors constitutively active transactivation function (AF-1) and multiple autonomous transactivation domains. This is followed by a DNA-binding domain (DBD, C domain), through which an activated NR binds to a DNA response element. The ligand-binding domain (LBD, E domain) at the carboxyl end encompasses the activation function-2 region (AF-2). A less conserved flexible hinge domain (D) connects DBD and LBD. The hinge region contains a nuclear localization signal (NLS) sequence, which extends to the 3' end of DBD. A variable F domain follows the LBD E domain in some but not all NRs. X-ray crystallography, cryo electron microscopy and solution structure determination by various methods including small-angle X-ray scattering and hydrogen-deuterium exchange, revealed DBD and LBD structures of several NRs, such as the first and second zinc finger modules and DNA-binding specificity motif of DBD; receptor dimerization motif; twelve α-helices of LBD and ligand-induced helix-12 repositioning that creates an interaction surface for coactivator or corepressor recruitment (*Pawlak et al, 2012*; *Helsen & Claessens, 2013; Carlberg & Campbell, 2013*).

DNA elements cognate to Type I-III NRs constitute repeats of the half-site consensus sequence RG(G/T)TCA (R: purine), configured as a direct repeat (DR), inverted repeat (IR), or everted repeat (ER) and separated by a varying number of nucleotides. Type I NRs recognize IR3-type palindromic elements; Type II and III NRs recognize specific repeat motifs of the consensus half site. Type IV NRs bind to a single hexamer consensus RG(G/T)TCA, which may contain a short preferred sequence 5' to the hexameric site (*Mullican et al, 2013*).

Preferred response elements for PXR, CAR and VDR are 3- or 4- nucleotide spaced direct repeats (DR3, DR4), as concluded from *in vitro* DNA-binding studies and response element-induced promoter activity in transfected cells. Numerous PXR/CAR/VDR target genes are also found to contain ER or IR

motifs as response elements. Nevertheless, genome-wide chromatin immunoprecipitation (ChIP) and deep sequencing of immunoprecipitated DNAs (ChIP-Seq) identified DR4 as the most frequent PXR-associated recruitment sites in mouse liver (*Cui et al, 2010*). DR4 in the human genome is a preferred DNA-binding site for the CAR/RXR heterodimer as well, as recently observed in a modified yeast one-hybrid assay (*Hosoda et al, 2015*). DR3 is the prevalent VDR-binding site at genomic regions that contain primary VDR target genes. These genomic regions are induced for chromatin opening in response to 1,25-D_3 signaling (*Seuter et al, 2014*).

2a. ***Regulation of PXR, CAR, VDR expression***

PXR and CAR are the primary mediators of transcription regulation of ADME relevant genes. Pathological conditions negatively impacts drug metabolism due to reduction of PXR and CAR activity. As an example, CYP3A4 expression is suppressed by inflammation in part due to interference of inflammation-activated NF-κB with PXR's transactivation function, since the p65 subunit of NF-κB was found to disrupt DNA binding of the PXR/RXRα complex in the *CYP3A4* gene (*Gu et al, 2006*). Reduced PXR and CAR activity impairs drug metabolism under conditions of hepatic steatosis as well, since SREBP-1 (sterol regulatory element binding protein-1), activated in hepatocytes by lipogenesis-stimulated LXR-α, prevented p160 coactivator interaction with CAR or PXR, which curtailed phenobarbital-induced, PXR/CAR-mediated *CYP3A4/CYP2B6* gene transactivation (*Roth et al, 2008*).

PXR and *CAR* gene expression is regulated by many transcription factors including various NRs (*Aouabdi et al, 2006; Kumari et al, 2012; Yang & Wang, 2014*). Cholic acid-activated FXR robustly induced the mouse *Pxr* gene in the liver via four FXR-binding elements in the *Pxr* promoter (*Jung et al*, 2006). HNF4α regulates xenobiotic response in mice during fetal liver development through *Pxr* gene activation (*Kamiya et al, 2003*). GR regulated rat *Pxr* promoter in transfected primary hepatocytes and

in hepatoma cells (*Shi et al, 2010*). Human PXR expression in liver is transcriptionally regulated by PPARα (*Aouabdi et al, 2006*) and HNF4-α (*Iwazaki et a, 2008*). Expression of CAR is induced by agonist ligands for GR, PPARα and functional binding sites for these NRs as well as a binding site for HNF4α were identified in the upstream sequence of the CAR promoter (*Ding et al, 2006*; *Yang &Wang, 2014*). Furthermore, in animal studies, CAR mRNA expression was induced by fasting and calorie restriction (*Ding et al, 2006*). Additional mechanisms entailing genetic polymorphism, changes in the epigenetic landscape, post-transcriptional regulation by micro RNAs, and functional modulation through posttranslational modification (PTM) can have major impacts on the expression and activity of PXR and CAR. These examples are discussed under sections 3a and 3b.

VDR, upon activation by cognate ligands (i.e. 1,25-D_3 and lithocholic acid, LCA), can also induce ADME relevant genes, especially in the intestine. Examples for VDR-mediated induction of DMEs and drug transporters in 1,25-D_3- or LCA-treated cells include *CYP3A4*, *CYP2B6*, *CYP2C9* (*Makishima et al, 2002*; *Drocort et al, 2002*), *SULT2A1* (*Echchgadda et al, 2004*), *OATP1A2* (*Eloranta et al, 2012*), *ABCA1* (*Tachibana et al, 2009*), and *MRP3*, *MRP2* (*Fan et al, 2009*). Crystal structures of VDR bound to LCA- and 3-ketoLCA have been determined (*Masuno et al, 2013*). Seasonal differences in intestinal CYP3A4 levels are attributed to season-related fluctuations in sunlight exposure that leads to variations in serum levels of 25-hydroxy-D_3 and 1,25-D_3 (*Thirumaran et al, 2012*).

Transcription of the *VDR* gene is under auto-regulation; 1,25-D_3 can increase *VDR* gene expression. Various endocrine factors including parathyroid hormone, retinoic acid, and glucocorticoids also regulate *VDR* expression (*Pike & Meyer, 2010*). Like PXR/CAR, VDR expression/activity is influenced by gene polymorphism, micro RNAs and by post-translational modification, as discussed in section 3.

2.b. ***Xenobiotic response element (XRE)****:*

At the chromatin level, XREs serve as sensors of xenobiotic (or endobiotic) signals by recruiting activated PXR/RXR and CAR/RXR to target genes. XRE activation is demonstrated by its activity in cis to induce promoter-directed reporter gene expression in transfected cells. Screening for XRE activation by synthetic or semi-synthetic chemicals is an integral part of the workflow for drug development. XREs also help identify other regulatory factors, which modulate PXR- and CAR-mediated expression of phase 0-III mediators. XREs in the phase II DME genes *UGT1A1* (for the glucuronidating enzyme UDP glucuronosyltransferase, isoform A1, subfamily-1) and *SULT2A1* (for the sulfotransferase enzyme, isoform A1, subfamily-2) are briefly described:

The phenobarbital responsive enhancer module (PBREM) in the human *UGT1A1* includes three CAR-responsive XREs that are required for the optimal induction of *UGT1A1* by phenobarbital (PB) (*Sugatani et al, 2001*; *Frank et al, 2003*). Protein-DNA interaction, analyzed by electrophoretic gel mobility shift assay (EMSA), revealed that CAR binds as a monomer to one of the functional XREs in the *UGT1A1* PBREM, and similar to the CAR/RXR dimer, the DNA-bound CAR monomer can interact with coactivators and corepressors. Furthermore, binding of the monomeric CAR or CAR/RXR dimer to XRE is most favored when the hexamer repeat of the response element is preceded at the 5' end by the dinucleotide AG. Arginine residues at positions 90 and 91, located within the carboxy-terminal extension of CAR's DBD, mediate the dinucleotide-dependent binding preference (*Frank et al, 2003*).

XRE-dependent and PXR- and CAR-mediated induction of human *SULT2A1* was investigated in our laboratory (*Echchgadda et al, 2007*). Preferred substrates for SULT2A1 are bile acids and dehydroepiandrosterone (DHEA) -- the latter is the steroid precursor for testosterone and dihydrotestosterone. A major role for SULT2A1 in the enterohepatic tissue is to promote bile acid clearance as the sulfate conjugate. Notably, the prostate cancer drug Zytiga® (abiraterone acetate) is

hydrolyzed *in vivo* to the therapeutic metabolite abiraterone, which is cleared from the body after conversion by SUL2A1 to the inactive abiraterone sulfate, and by CYP3A4 to the inactive N-oxide abiraterone, which is then converted to a sulfated derivative (*PubChem database, CID 132971*). *SULT2A1* expression is induced by VDR and the bile acid receptor FXR as well, which is in keeping with its role in bile acid homeostasis (*Song et al, 2001*; *Echchgadda et al, 2004*). A PXR/CAR-responsive composite XRE in the human *SULT2A1* promoter and a synergizing role of HNF4-α in XRE-induced *SULT2A1* expression is described below and is summarized schematically in **Figure 3**.

A composite XRE and HNF4-α-responsive DR1 element in the human SULT2A1 promoter:

Induction of the *SULT2A1* promoter by ligand-activated PXR and CAR in transfected liver and intestinal cells was shown to be mediated by an upstream xenobiotic-responsive composite element (XRE). Specific interaction of XRE with PXR/RXRα and CAR/RXRα was demonstrated by DNAse1 footprinting and EMSA. The XRE from -190 to -131 positions, was defined by an inverted repeat and a direct repeat of the AG(G/T)TCA element, which are configured as IR2 ($^{-190}$AACGCA**AGCTCA-**GA**TGACCC**CTAA^{-167}) and DR4 ($^{-155}$GATA**AGTTCA**TGAT**TGCTCA**ACATC^{-131}) (*Echchgadda et al, 2007*). XRE-mediated stimulation required both IR2 and DR4 elements; neither by itself was sufficient to cause robust *SULT2A1* promoter induction. Thus XRE is a composite element. The composite XRE spanning -190 to -131 positions stimulated a heterologous promoter. Point mutations in the XRE prevented its interaction with PXR and CAR and abrogated induction of the *SULT2A1* and the heterologous thymidine kinase promoter.

HNF4-α plays a modifying role in the PXR- and CAR-mediated target gene transcription, since HNF4-α potentiated PXR- and CAR-mediated transactivation of the *SULT2A1* promoter. A DR1 element ($^{-63}$GTGACATGC**TGGGAC**A**AGGTTA**AAGATCG^{-35}) in the *SULT2A1* gene promoter, located

upstream of -30 nucleotide position, serves as an HNF4-α-binding element. A schema on the regulation of *SULT2A1* by PXR and CAR via the composite XRE, and the synergizing influence of DR1-bound HNF4-α on xenobiotic-induced *SULT2A1* expression is presented as schema in **Figure 3**.

***ii) Sult2A1 induction by FXR via an IR0 element*:** Apart from xenobiotic chemicals, bile acid overload induces *SULT2A1* expression. For example, Sult2A1 mRNAs were induced in the mouse liver when animals were fed a cholic acid containing diet (**Fig. 4**), and bile acid activated FXR robustly induced the *Sult2A1* promoter through an FXR-bound IR0 element (*Song et al, 2001*). However, IR1 is the most abundantly encountered FXR-responsive element. An IR1 element drives FXR-mediated transactivation of *ABCB11*, the gene for the human bile salt export pump (*Plass et al, 2002*). A number of other repeat motifs of the half site RG(G/T)TCA including DR1, ER6, ER8 are known to be FXR-responsive functional elements in FXR target genes. Assessment of genome-wide FXR binding in the mouse hepatic chromatin showed an IR1-type sequence as the preferred chromatin occupancy site for FXR *in vivo* (*Chong et al, 2010*). FXR-occupied IR1 sites are frequently juxtaposed to a hexameric half-site consensus sequence, which binds a monomeric NR such as LRH-1 (liver receptor homolog-1). Positive interplay between FXR and LRH-1 for the gene encoding the small heterodimer partner (SHP), which is an atypical NR devoid of a DBD, as well as several other FXR target genes has been demonstrated (*Schapp et al, 2014*). The FXR/LRH-1/SHP axis plays a key role in bile acid homeostasis, as discussed in the next section.

In summary, above examples of XREs demonstrate that PXR, CAR, FXR bind a variety of repeat motifs of the consensus half site RG(G/T)TCA to induce genes involved in drug metabolism and disposition.

2.c. ***NR, a drug target for diseases from disrupted bile acid/cholesterol homeostasis*****:**

Bile acid synthesis is the primary pathway for cholesterol catabolism in liver, accounting for ~50% of daily cholesterol turnover. Cholesterol overload, the underlying cause for cholesterol stone, results from insufficient bile acid synthesis when bile acid saturation with cholesterol leads to the formation of cholesterol stone. On the other hand, bile acid accumulation leads to cholestasis due to reduction or stoppage of bile flow. Oral bile acid therapy is given to patients with cholesterol stones, and ursodeoxycholic acid is used to treat cholestasis of pregnancy and primary biliary cirrhosis (PBC), the autoimmune disease causing bile duct destruction. FXR and other NRs, such as LRH-1, HNF4-α, LXR-α, SHP, PXR and VDR maintain bile acid/cholesterol homeostasis (*Kir et al, 2012*; *Schaap et al, 2014*).

CYP7A1 (cholesterol 7α hydroxylase) is the rate-limiting enzyme for bile acid production from the catabolic breakdown of cholesterol. HNF4-α and LRH-1 are positive regulators of *CYP7A1* expression. Some aspects of CYP7A1 regulation are, however, species-specific -- a prominent example being the positive regulation of the basal expression of *CYP17A1* by oxysterol-activated LXR-α in the rodent liver but not in human liver, since the LXR-binding site in the human promoter is mutated (*Handschin et al, 2004*). Toxic accumulation of bile acids, on the other hand, is prevented by FXR-imposed negative feedback regulation of *CYP7A1*. In this case, bile acid activated FXR induces SHP (*Goodwin et al, 2000*), and interference from SHP due to protein-protein interaction inhibits positive regulation of the *CYP7A1* promoter by LRH-1 (an NR activated by phospholipids) (*Kir et al, 2012*). SHP also interferes with the stimulatory interaction between HNF4-α and the coactivator PGC1-α (peroxisome proliferator activated receptor γ coactivator 1-α) on the *CYP7A1* promoter (*Schaap et al, 2014*). In the ileum part of intestine, SHP plays a role in the *CYP7A1* repression by the fibroblast growth factor-19 which, like SHP, is induced by FXR (*Kir et al, 2012*). PXR blocks *CYP7A1* expression by disrupting the PGC1α →

HNF4 stimulatory axis (*Li & Chiang, 2005*). Thus, like FXR, PXR also regulates bile acid homeostasis upon activation by drugs and certain bile acids. Drugs targeting FXR, SHP, LRH-1, PXR and HNF4-α have therapeutic potential against liver and biliary disorders. Small molecules that augment SHP activity may robustly reduce *CYP7A1* expression to prevent bile acid overload. Small molecules, which elevate LRH-1 activity or increase PGC1-α ↔ HNF4-α interaction, would be useful in enhancing CYP7A1 expression, which then would promote cholesterol breakdown and reduce cholesterol build up.

Apart from FXR, TGR5, a transmembrane G protein coupled receptor, mediates bile acid signaling. TGR5 is located in intestinal epithelium, Kupffer cells, sinusoidal endothelium and bile duct cells. Both TGR5 and FXR are hotly pursued drug targets for diseases of errant bile acid and cholesterol metabolism (*Schaap et al, 2014*). The athero-protective effect of LXR-α arises in part from the LXR-α-mediated induction of efflux transporters in resident macrophages of the arterial wall, and this in turn promotes cholesterol efflux and reverse cholesterol transport to the liver and intestinal tissue and subsequent removal of cholesterol as part of excreta. Therefore, small molecule activators of LXR-α may normalize cholesterol homeostasis. Finally, VDR can regulate bile acid and cholesterol homeostasis, since agonist-activated VDR promotes cholesterol catabolism by repressing SHP and increasing CYP7A1 expression (*Chow et al, 2014*).

3. Genetics, epigenetics and interindividual differences in drug response

3.a. *Gene polymorphism and NR-regulated variable DME/drug transporter activity:*

Single nucleotide polymorphism (SNP) at regulatory loci of ADME related genes, or non-synonymous SNPs in the coding region of NR itself, alter NR-mediated DME/transporter expression. A non-coding SNP at an HNF4-α binding site in the *CYP2B6* promoter contributes to the interindividual variations in *CYP2B6* expression (*Lamba et al, 2003*), and a common African haplotype for an SNP at a

PXR-binding enhancer in *GSTA* (encoding glutathione S-transferase A) causes hypersensitivity for *GSTA* induction by the human PXR ligand rifampin (*Smith et al, 2014*). CYP2D6, which metabolizes a large number of drugs including antidepressants and β blockers, shows wide interindividual differences in expression and activity. An HNF4-α variant having reduced binding to the *CYP2D6* promoter and causing decreased *CYP2D6* expression has been identified. The variant HNF4-α arises from a non-synonymous SNP, which yields glycine→aspartic acid substitution at the position 60 (G60D). Compared to this variant, the wild-type HNF4α genotype is associated with higher CYP2D6 activity in the human liver (*Lee et al, 2008; Huang-Verslues & Sladek, 2010*). The G60D HNF4-α appears at low frequency in Asian populations; it has not been detected in Africans or Caucasians (*Lee et al, 2008*). Variable *CYP2D6* expression also results from gene amplification that ranges from 3 to 13 gene copies. CYP2D6 deficiency is an autosomal recessive trait in ~7% Caucasians and ~1% Orientals, making these individuals poor metabolizers of CYP2D6 drug substrates (*Bertillson et al, 2002*). Pharmacogenomic tests for CYP2D6 variants are common practice for assessing the appropriateness and efficacy of a CYP2D6 drug substrate. Interindividual differences in drug response are managed by dosage adjustment based on the patient's pharmacogenetic profile.

The basal level of CYP3A4 in the liver varies up to 60-fold between individuals, although SNPs in coding sequences and regulatory loci of *CYP3A4* do not explain this variability (*Hustert et al, 2001*). Association analysis suggests that non-synonymous SNPs of PXR and FOXA2 (aka HNF3-β, a liver-enriched transcription factor) contribute to CYP3A4 variation in the human liver, since the mRNA expression level for CYP3A4 in the human liver significantly relates to SNPs of PXR and FOXA2, and PXR expression itself is regulated by FOXA2. Binding sites for FOXA2 and PXR in the human *CYP3A4* distal promoter were identified (*Lamba et al, 2010*). VDR polymorphism accounts for disparate intestinal CYP3A4 levels and variable first pass intestinal absorption and metabolism of CYP3A4-targeted drugs *(Thirumaran et al, 2013*).

FXR, which regulates the expression of many uptake and efflux transporters, shows a common non-coding -1G>T polymorphism, where T replaces G at the -1 position of the translation start site causing reduced FXR expression. The FXR-1G>T SNP is associated with increased efficacy of the statin drug rosuvastatin in lowering hepatic cholesterol biosynthesis, thus affording greater LDL-cholesterol response (*Hu et al, 2012*). Rosuvastain remains un-metabolized in hepatocytes and ABCG2 (BCRP), an apical ABC cassette efflux transporter, plays a major role in the biliary clearance of rosuvastatin. ABCC2 (MRP2) and possibly ABCC11 (BSEP) also contribute to rosuvastatin disposition from human liver. Mechanistically, low expression of the variant FXR accounts for reduced expression of the transporters ABCG2, ABCC2, ABCC11, which leads to a blockade in the biliary clearance of rosuvastatin and longer residency of the drug in hepatocytes – hence a more potent effect of this statin on hypercholesterolemic patients who carry the FXR-1G>T SNP (*Hu et al, 2012*).

A large number of SNPs for PXR (NR1I2)- and CAR (NR1I3)- encoding genes are known, several of which are associated with altered expression and/or function of these receptors (*Schwabedissen & Kim, 2009*; *Swart et al, 2012*). For the *NR1I2* SNP 63396C>T, located in a putative transcription factor binding site, the 63396T variant associates with elevated PXR expression, increased *CYP3A4* expression and decreased plasma levels of the CYP3A4 substrate atazanavir (an anti-retroviral drug). Natural PXR variants, which harbor single amino acid changes, confer altered transactivation response of the *CYP3A4* promoter (*Hustert et al, 2001*). Among the 22 naturally occurring splice variants of CAR, some are non-functional due to nonsense mutations. For the *CAR* (*NR1I3*) SNP rs2307424C>T, the T allele is associated with a low plasma level of the anti-retroviral drug efavirenz, which is a CYP2B6 and CYP3A4 substrate (*Wyen et al, 2011*). Extensive *VDR* gene polymorphism has been reported (*Uitterlinden et al, 2004*), and it has been reported that intestinal CYP3A4 expression levels are functions of *VDR* polymorphisms (*Thirumaran et al, 2012*).

3.b. ***Epigenetic machinery and drug response***

Roles for DNA methylation, histone modification and microRNAs in the regulation of a large number of mediators of phase 0-III processes and their NR regulators (PXR, VDR, HNF4-α) have been reported (*Ingelman-Sundberg et al, 2013*; *Ivanov et al, 2014*). Epigenetic factors confer heritable changes in chromatin structure and function, caused by mechanisms other than DNA sequence alteration at the coding or non-coding region of a gene. An integral role of epigenetics in health and disease is revealed by the tragic history of the synthetic estrogen diethyl stilbesterol (DES) as a birth control pill. *In utero* DES exposure caused vaginal tumors and breast cancer in adult females. In mice, DES altered gene-specific DNA methylation, expression of epigenetic enzymes (DNMT3A, MBD2, HDAC2, EZH2), and the abundance of HOTAIR, a lncRNA (*Nilsson & Skinner, 2014*). Epigenetic systems are briefly discussed and current knowledge on their roles in drug metabolism and drug response is presented.

3.b.1. ***DNA methylation, ADME gene activity, interindividual differences***: DNA methylation at the 5' cytosine of the CpG sequence (5mC) is an epigenetic mark for gene activity (*Sharma et al, 2010*). Gene repression is linked to hypermethylated promoters when 5mC methylation occurs within long stretches of CpG repeats (CpG island) at proximal promoters, although 5C-methylation at low CpG density (CpG shores) or even at single CpG sites can mark reduced gene expression. Of 3 major DNA methyltransferases (DNMTs) in mammals, DNMT1 is the maintenance methyltransferase; DNMT-3a and -3b are *de novo* enzymes, essential for the genome-wide methylation of DNA following embryo implantation. Gene repression by DNA hypermethylation is aided by the interaction of DNMTs with the polycomb repressor complex (PRC2), especially with EZH2 (Enhancer of Zeste homolog 2), the histone methyltransferase component of PRC2 (*Sharma et al, 2010*). Cancer development is associated with genome-wide DNA hypomethylation, which activates proto-oncogenes. For many tumor suppressors, site-specific hypermethylation contributes to gene silencing (*Sharma et al, 2010*). 5-

hydroxymethylcytosine (5hmc) modification of DNA, on the other hand, is an activation mark, linked to active gene transcription (*Ivanov et al, 2010*). Notably, the paternal sperm DNA methylation pattern has been linked to autism risks in an autism-dense cohort (*Feinberg et al, 2015*). Extensive interindividual differences in the genome-wide DNA methylation pattern have been reported (*Zhang et al, 2010*).

Acquired drug resistance has been linked to altered DNA methylation of NRs and NR-regulated ADME genes, as observed in i) drug-induced demethylation of *MDR-1* and *BCRP*, which leads to their overexpression causing multidrug resistance (MDR) of cancer cells (*Bram et al, 2009; Ivanov et al, 2012*); ii) drug-induced methylation of the estrogen receptor (ER-α) encoding *ESR1* gene promoter, causing reduced ER-α expression and tamoxifen resistance in breast cancer (*Pathiraja et al, 2010*); and iii) resistance to progesterone therapy in endometrial cancer due to reduced expression caused by enhanced methylation of the gene encoding progesterone receptor isoform A (PR-A) (*Shao, 2013*). Methylation of the *PXR* gene promoter attenuated PXR expression and reduced *CYP3A4* expression in colon cancer cells (*Kacevska et al, 2012*). In colon and endometrial cancers, the *VDR* gene is aberrantly methylated (*Kacevska et al, 2012*); differential methylation of *PXR* and *FXR* at CpG promoter sites has been reported in cholestatic pregnancy versus normal healthy pregnancy (*Cabrerizo et al, 2014*).

A role for DNA methylation in the expression of a number of DMEs and drug transporters has been reviewed (*Gomez & Ingelman-Sundberg, 2009; Ivanov et al, 2014***).** A few representative examples are discussed here. 1) *CYP3A4/5/7* expression is dependent upon the methylation status of these genes, since their expression was altered when human hepatoma cells were treated with 5-aza-2′-deoxycytidine (a DNA demethylating agent). *CYP3A4* induction is associated with reduced 5mC at CpG-rich regions located at or near the binding sites for PXR, CAR and VDR, the well-known regulators of *CYP3A4* (*Ivanov et al, 2014*). 2) Altered *CYP1A1* expression in response to cigarette smoking is associated with changes in the methylation status of *CYP1A1*. 3) Development stage-dependent *CYP2E1* expression is

influenced by the methylation status of this gene. 4) Phase II genes including *UGT1A1*, *GSTP1*, *SULT1A1* and genes for efflux transporters like MDR1, BCRP and members of the OATP family of uptake transporters are epigenetically regulated due to DNA methylation (*Reed et al, 2009*; *Imai et al, 2013*; *Imai et al, 2013*; *Xie et al, 2014*).

3.b.2. ***Histone marks; impact on*** **NR-regulated** ***ADME genes*****:** Post-translational modification (PTM) of histones (methylation, acetylation, phosphorylation, ubiquitinylation, sumoylation, ADP-ribophosphorylation and several other modifications), especially acetylation and methylation at the amino-terminal histone tails for histone H3 and H4, are well-characterized epigenetic signatures that influence gene activity. PTMs are also known for histone H2A and H2B and the linker histone H1. More than 10 different PTMs at ~80 sites on histone tails, histone core domains and on the H1 linker histone have been identified (*Cohen et al, 2011*). Gene-activating histone marks include H4 lysine-16 acetylation (H4K16ac); H3 trimethylation at lysine-4 (H3K4me3) and lysine 36 (H3K36me3), and H3 phosphorylation at serine-10. Among repression marks, trimethylated histone H3 at lysine-9, lysine-27 and lysine-20 are most well characterized (*Kouzarides, 2007*). Histone deacetylases (HDACs, subgrouped as class I to IV), histone acetyltransferases (HATs), histone methyltransferases (HMTs) and histone demethylases (HDMs) are important drug targets. Numerous lysine-specific HMTs (SET7/9, MLL, EZ2H2) and lysine-specific HDMs (LSD1/KDM-1, JMJD2A/KDM4A, JARID1A/KDM5A) have been characterized. Arginine methylation of histones mediated by protein arginine methyltransferases (PRMTs) also regulates gene activity, as seen for histone H3 Arg-17, H4 Arg-3. Histone marks are the "codes" that are "read" by chromatin remodelers (such as SWI/SNF containing complexes) and histone-modifying enzyme complexes (such as PRC2) in order to prepare chromatin for positive or negative transcriptional response. Enzymes that mark histones through PTM are "writers"; those involved in

removing histone marks are “erasers”; and protein/enzyme complexes, which recognize histone codes, are “readers”. Crosstalk of HMTs with DNMT1 influences epigenetic regulation (*Sharma et al, 2010*).

Among the ADMEs whose genes are known to be regulated by histone modification include the phase I DMEs CYP3A4, CYP2E1, phase II DMEs SULT2B1, UGT1A1, the efflux transporters MDR1, BCRP, the OATP family of uptake transporters and the *SLC5A5* encoded iodine uptake transporter sodium/iodide symporter (*Imai et al, 2013*; *Ivanov et al, 2014*). Histone modification can have long-lasting effects on ADME genes. The CAR target genes *Cyp2b10* and *Cyp2c37* remained induced in adult mouse livers and adult mice were much less sensitive to zoxazolamine (Cyp2b10 substrate), when at neonatal life they were exposed to the CAR ligand TCPOBOP. H3K4 methylation and H3K9 demethylation at CAR-responsive elements in the *Cyp* genes was maintained in the adult mouse liver and played a role in *Cyp* gene induction (*Chen et al, 2012*). PXR-mediated *CYP3A4* induction was regulated by PRMT1, which methylated histone H4 at arginine-3, which is located within a PXR-responsive chromatin region in the CYP3A4 gene (*Xie et al, 2009*). In a rat model of chronic kidney disease, reduced *Cyp2C* and *Cyp3A* expression, and associated reduction in PXR and HNF4-α binding to cognate sites in the *Cyp2C11* and *Cyp3A2* promoters, was accompanied by reduced histone H4 acetylation at the *Cyp3A2* promoter regulatory region and reduced histone H3 acetylation at the PXR- and HNF4-α-bound regulatory loci of *Cyp2C11* and *Cyp3A2* promoters (*Velenosi et al, 2014*).

Given the reversible nature of chromatin modifications by DNA methylation and histone PTM, drugs targeting epigenetic enzymes (“epi drugs”), especially DNMT, HAT, HDAC, HMT and HDM, are being developed. 5-azacytidine (Azacitidine) and 5-aza-2’-deoxycytidine (Decitabine) are nucleoside analogs and DNA demethylating agents that are clinically used against myelodysplastic syndromes,

chronic myelomonocytic leukaemia and acute myeloid leukaemia. Second-generation DNA de-methylating agents (SGI-110, CP-4200) are under development (*Jordheim et al, 2013*).

Valproic acid, a class I HDAC inhibitor and an anticonvulsant, activates CAR and PXR to induce CYP3A4, CYP2B6 and MDR-1 expression (*Cerveny et al, 2007*; *Takizawa et al, 2010*; *Ni et al, 2015*). Valproic acid also enhances tissue sensitivity to estrogen and progesterone by potentiating estrogen receptor (ER) and progesterone receptor (PR) activity due to HDAC1 inhibition (*Jansen et al, 2004*). Vorinostat (a hydroxamate) and romidepsine (a depsipeptide) are orally administered pan-HDAC inhibitors, which are used in combination therapy with chemotherapeutics like paclitaxel, doxorubicin. Vorinostat resistance is thought to develop from increased expression of efflux transporters, since MDR-1, BCRP and MRPs were detected at elevated levels in vorinostat-treated cells (*Ivanov et al, 2014*). Inhibition of ABC transporters in this case may improve vorinostat's therapeutic efficacy. Epi drugs, which target histone methylation, are at various stages of development (*Wei et al, 2011*).

3.b.3. ***Non-coding RNA-mediated regulation of PXR, CAR, VDR and ADME gene expression***:

Non-coding RNAs (ncRNAs), best characterized for micro RNAs, are integrally linked to epigenetic machinery. Transcripts of more than 90% of the human genome represent ncRNAs, many of which regulate gene expression at transcriptional and posttranscriptional levels. Short (<30 nucleotides) ncRNAs, best characterized for micro RNAs (miRNAs), and long ncRNAs (lnc RNAs) with > 200-nucleotide lengths are two major categories of ncRNAs. The list for micro RNAs, which influence ADME gene expression, is steadily growing (*Kacevska et al, 2012*; *Yu & Pan, 2012*; *Rieger et al, 2013*).

The miRbase lists as many as 2555 unique mature human miRNAs (database *version 20; June 2013 release*). Base pairing of the miRNA nucleotide sequence with a cognate sequence in the 3'

untranslated region (3'-UTR) of a target messenger RNA (mRNA) within the RNA-induced silencing complex leads to either mRNA degradation (in the case of a perfectly complemented base pairing), or translational suppression of the target mRNA when base pairing is not 100% complementary. A single miRNA can target 3'UTRs of multiple messenger RNAs.

Studies in cell culture show that the messenger RNAs for ADME related genes are targeted directly or, via upstream regulatory NR and other transcription factors, by one or more miRNAs. Regulation of CYP1B1 and CYP3A4 mRNAs by miR-27b; CYP2E1 mRNA by miR-378; the MDR1 transporter by miR-451; and the BCRP transporter by miR-328, miR-519C, miR-520h underscores the impact that miRNAs may have on drug metabolism and disposition, provided these miRNA-dependent regulations are upheld *in vivo*. While miR-27b directly regulates CYP3A4 expression, the VDR level is regulated by this micro RNA as well, so that miR-27b can both directly and indirectly influence CYP3A4 expression. PXR expression is regulated also by miR-148a. The miRNA-dependent regulation of several epigenetic enzymes including DNMTs, HDACs, EZH2, and epigenetic enzymes that regulate miRNA-specifying genes (which produce miRNA precursor transcripts) have been reported (*Chung & Jones, 2007*; *Wang et al, 2014*). Such cross talks provide a miRNA-dependent additional regulatory cascades that may alter DME/transporter expression. The abundance of specific miRNAs may predict drug response, since miR-21 levels in pancreatic cancer biopsies correlated with gemcitabine responsiveness, and ectopic miR-21 expression caused gemcitabine resistance in pancreatic cancer cells (*Giovanetti et al, 2010*).

A long non-coding RNA (lncRNA), known as AIR, is indirectly involved in the inactivation of the mouse organic cation transporter (OCT) genes *Slc22a2* and *Slc22a3*, since AIR plays a role in silencing the *Igf2R* gene cluster and *Slc22a2* and *Slc22a3* are located within this cluster (*Sleutels et al, 2002*). LncLSTR, a recently reported liver-enriched lncRNA, is a regulator of *Cyp8b1*, which is involved in bile acid biosynthesis (*Li et al, 2015*). The lncRNAs PCA3 and PCGEM1 are elevated in human prostate

cancer. PCGEM1 coactivates activities of the androgen receptor and cMYC oncoprotein. (*Hung et al, 2014*). Whether lncRNAs directly regulate ADME-relevant genes remains to be determined.

4. Drug interactions: a role for xenosensing NRs

4.a. *Drug-drug, drug-food, drug-herb interaction*

Drug-drug interaction (DDI) reflects changes in target drug pharmacokinetics or bioavailability in the presence of a co-administered drug. By activating PXR and CAR, the interfering drug renders changes in one or more components of the drug metabolizing and disposition machinery. DDI is assessed quantitatively by the pharmacokinetic parameters C_{max}, which refers to the peak plasma drug concentration at post-dosing; and AUC (area under the time-plasma drug concentration curve), which defines total serum drug levels over time. DDI has three possible outcomes: i**)** overdosing and potential toxicity due to increased half-life of a target drug caused by one or more of the following -- excessive pro-drug bioactivation; attenuated DME activity; increased uptake activity and reduced efflux activity of transporters; ii**)** underdosing resulting in low drug efficacy, which is due to reduced drug uptake and/or reduced bioactivation; enhanced metabolism and/or accelerated drug efflux; iii**)** a boost in medicinal potency. CYP-mediated DDI led to the withdrawal of numerous drugs from clinical use, such as terfenadine (the antihistamine Seldane®) and cerivastatin (a cholesterol-lowering statin). Dietary ingredients (e.g. furanocoumarins in grapefruits/grapefruit juice) or phytochemicals in medicinal herbs (e.g. hyperforin in St John's Wort) can modulate a drug's efficacy and engender potentially fatal drug-food and drug-herb interactions. CYP3A4/3A5 and CYP2D6 are most frequent participants in DDI (*Lynch & Price, 2007*; *Shirasaka et al, 2013*).

Desirable outcomes may also result from drug interactions, as seen in the hepato-protective effect of ginger extracts against diverse drugs including high-dose acetaminophen (*Haniadka et al, 2013*). DDI is not a concern for peptide or antibody based therapeutics, since they do not activate PXR and CAR.

Recently approved PCSK9 inhibitors are antibody-based drugs, which aid in LDL-cholesterol clearance from circulation by preventing PCSK9-mediated degradation of the LDL receptor (*Roth* et *al, 2012*).

4.b. *Linking PXR, CAR to drug interactions*

PXR and CAR activities are closely linked to DDI (*Willson &Kliewer, 2002*). Apart from acting as direct ligands, certain drugs induce phosphorylation of PXR and CAR by activating signal pathways that lead to activation of kinases such as PKA, PKC, CDK2, CDK5 and p70S6K (*Smutny et al, 2013*). Forskolin, a diterpene constituent of the Indian plant *C. forskohlii,* is used for the treatment of glaucoma, asthma and various other diseases. Forskolin induces PXR phosphorylation through PKA activation, and enhances PXR-coactivator interaction upon its direct binding to the PXR LBD (*Staudinger, 2006*). Additionally, forskolin is a constituent of an herbal mixture marketed over-the-counter for weight loss. DDI/drug-herb interaction may interfere with forskolin's therapeutic value.

Metformin induces phosphorylation of CAR at threonine-38, which results in the inhibition of CAR's nuclear translocation and CAR-mediated induction of target genes such as *CYP2B6*. Co-administered metformin alters the pharmacokinetics of CYP2B6 drug substrates (*Zamek -Gliszczynski et al, 2014*). Metformin-induced phosphorylation of CAR is mediated by activated AMPK and the MAP kinase ERK1/2. Also, metformin disrupts interaction of CAR with coactivators (*Yang et al, 2014*). Additionally, metformin clearance will be prevented in the event that CYP2B6-targeted drugs interfere with the renal OCT2/MATE transporter system. In this case, the negative interplay of metformin with CAR-induced *CYP2B6* expression will be amplified leading to a markedly pronounced DDI.

A few representative examples are presented here to highlight the central role of PXR and CAR in clinically significant drug interactions. NR-regulated drug interactions are comprehensively discussed in many reviews (*Harmsen et al, 2007*; *Neuvonen, 2010*; *König et al, 2013; Yu et al, 2015*).

4.c. ***Drug-drug interaction***

i) Statin metabolism, DDI and myopathy: involvement of transporters and DMEs: Statins reduce hepatic cholesterol synthesis by inhibiting HMGCoA reductase, hence the cholesterol-lowering activity of this class of drugs. Several uptake transporters of the OATP family (OATP1B1, OATP1B3, OATP2B1) are predominantly involved in the import of statins into hepatocytes. A short form of OATP2B1, expressed in human liver, shows robust importing activity for many drugs and endobiotic molecules including rosuvastatin and estrone sulfate (*Knauer et al, 2013*). The apical efflux transporters BCRP along with P-gp (MDR1) and MRP2 mediate extrusion of statin/statin metabolite out of cells. Like DMEs, the basal and induced expression of uptake and efflux transporters is regulated by PXR and CAR (*Tirona, 2011*). PXR- and CAR-responsive functional XREs have been characterized in genes for many drug transporters including OATPs, P-gp/MDR1, MRP2 and BCRP (*Tirona, 2011*).

Statins encounter adverse DDI with many common drugs. Cyclosporin A (an immune suppressant and OATP1B1substrate), competitively inhibits OATP-mediated uptake of pitavastatin, rosuvastatin by hepatocyts. This lowers therapeutic indices of these statins and leads to statin overload in the skeletal muscle, causing muscle pain or muscle weakness. Extreme statin overload causes rhabdomyolysis, a fatal condition that leads to renal failure and other adverse events. AUC for atorvastatin was elevated more than 600% in healthy volunteers when a single dose of rifampin was co-administered with the statin. Rifampin-induced inhibition of OATP for atorvastatin caused elevated AUC (*Lau et al, 2007*). Long-term rifampin treatment, however, reduced atorvastatin bioavailability due to induced expression of CYP3A4 and efflux transporters by rifampin-activated PXR.

Various statins significantly differ in pharmacokinetic characteristics due to differences in ADME. Atorvastatin, lovastatin and simvastatin are CYP3A4 substrates and thus, drugs inhibiting CYP3A4 (such as diltiazem, a blood pressure lowering drug) significantly raise the plasma levels of the above three statins; fluvastatin, a CYP2C9 substrate, does not interact with diltiazem. Pravastatin, rosuvastatin

and pitavatatin are eliminated from hepatocytes mostly in unmetabolized forms (pravastatin is resistant to CYP-mediated metabolism; rosuvastatin and pitavastatin are minimally metabolized). CYP3A4 inhibition does not cause DDI in this case (*König et al, 2013*).

ii) Prostate cancer, ZYTIGA®, CYP3A4-, CYP2C8-mediated DDI: Zytiga®, a CYP3A4 substrate, is an anti-androgen used to treat recurrent metastatic prostate cancer. Zytiga® exposure decreased by 55% in the presence of rifampin, indicating a need for higher Zytiga dosage when a PXR activator is co-administered (*Beckett et al, 2012*). Since Zytiga® inhibits CYP2C8, plasma levels of pioglitazone® (a CYP2C8 substrate and blood sugar controlling drug) increased when co-administered with Zytiga in a DDI trial on healthy subjects (*Clinical Pharmacol* 12.3; *FDA Drug Safety Reporting, 2015*).

4d. ***Drug-food interaction***

i) Grapefruit juice, drug transporters, PXR/CAR: Grapefruit/its juice contains a mixture of phytochemicals, most prominent being furanocoumarins and flavonoids, which inhibit OATPBs, CYP3A4, P-gP, MRP-2. Furanocoumarins/flavonoids interact with drugs such as cyclosporine that are substrates for components of drug metabolizing and transport machinery (*Hanley et al, 2011*).

ii) Alcohol, CYP2E1, Acetaminophen toxicity: CYP2E1, which metabolizes acetaminophen to highly reactive NAPQI, is strongly induced by drinking alcohol. Thus, Tylenol® (acetaminophen) can trigger adverse drug reaction if taken for an extended period along with continued drinking.

iii) Pomegranate juice, SULT2A1, Zytiga activity: Punicalagin, a polyphenol constituent of pomegranate juice, impairs sulfoconjugation of drugs in the intestine (*Saruwatari et al, 2008*). This inhibits clearance of orally delivered Zytiga which, as a CYP3A4 and SULT2A1 substrate, is normally metabolized from abiraterone acetate to abiraterone sulfate and *N*-oxide abiraterone sulfate, generated by CYP3A4-mediated oxidation and SULT2A1-mediated conjugation. Clearance of Zytiga is reduced because of its interaction with polyphenols in pomegranate juice, causing overdosing of the drug.

4.e. ***Drug-herb interaction***

i) St. John's Wort, PXR, CAR, CYP3A4: The phytochemical hyperforin, which confers the anti-depressant activity of St. John's wort, is a ligand for human PXR and CAR (*Moore et al, 2000; Chang, 2009*). Hyperforin-activated PXR/CAR induces *CYP3A4*, other *CYP* genes (*CYP2B6, CYP2C9, CYP2C19*), as well as *MDR1*. Acute rejection of transplanted hearts in patients due to self-medication with St. John's Wort is an example of serious drug-herb interactions. Rejection was caused by a drop in plasma levels of cyclosporine, which is a CYP3A4 and MDR-1 substrate (*Ruschitzka et al 2000*).

ii) Garlic, CYP2C9, the anticoagulant Warfarin: Warfarin is a CYP2C9 substrate. The organo-sulfer constituents of garlic inhibit metabolism of Warfarin by reducing CYP2C9 expression and activity. This increases systemic exposure to Warfarin and enhances the possibility for uncontrolled bleeding.

iii) Acetaminophen toxicity: a protective role of garlic via CAR-induced SULT: Hepato-protective effects of organo-sulfers in garlic extracts against acetaminophen-induced liver injury are due to two mechanisms: 1) reduced hepatic CYP2E1 expression and inhibition of CYP2E1-mediated acetaminophen biotransformation to a toxic metabolite (*Park et al, 2002*); 2) increased acetaminophen clearance as a sulfate metabolite by SULT activity. CAR, activated by diallyl sulfide (a garlic constituent) induces SULT2A1 and other SULTs (SULT1A1, SULT1A3/4, SULT1E1), promoting acetaminophen conversion to a sulfated metabolite (*Adjdi et al, 2008*; *Sueyoshi et al, 2011*; *McGill and Jaeschke, 2013*). Reduced acetaminophen build up prevents GSTpi induction by acetaminophen-activated CAR. This lowers oxidative stress from glutathione depletion and prevents oxidant-induced liver injury (*Zhang et al, 2002*).

Additional NRs can potentially generate drug interactions. VDR-mediated regulation of DMEs and transporters and a modifier role of HNF4 in the expression of ADME-relevant genes have been reported (*Makishima et al, 2000*; *Echchgadda et al, 2004*; *Echchgadda et al, 2007*; *Tirona, 2011; Knauer et al,*

2013). Whether long-term use of vitamin D supplements would cause adverse drug interactions should be evaluated. Drug interaction from activated glucocorticoid receptor (GR) is a distinct possibility, since ligand-activated GR induces CAR and PXR expression; a GR-responsive element has been identified in the CAR gene promoter (*Pascussi et al, 2003*). Dexamethasone, a synthetic glucocorticoid, promotes nuclear translocation of CAR and PXR and induces PXR/CAR target genes (*Pacussi et al, 2003*; *Sugatani et al, 2005*). Ketoconazole, an anti-fungal agent and GR antagonist, prevented rifampin- and phenobarbital-mediated PXR/CAR activation and induction of their target genes (*Duret et al, 2006*). Under ketoconazole co-medication, a primary drug may respond with altered pharmacokinetics.

4.f. ***Platforms for screening drug candidates****:*

Early assessment of drug candidates can avoid late-stage failure of clinical trials due to DDI and help minimize costs for developing and marketing a new drug. Candidate drugs are routinely screened in a cell based workflow for their impact on DME activity and PXR/CAR-mediated transactivation of XREs. Humanized mouse models, where *Pxr*, *Car* and *Cyp* rodent genes are replaced by corresponding human genes, are better suited for drug testing since these models provide *in vivo* relevance and they approximate as human surrogates (*Xie et al, 2000*; *Cheng et al, 2011*). A new hPXR-hCAR-hCYP3A4/3A7-hCYP2C9-hCYP2D6 mouse strain, having human PXR and CAR genes substituted for the rodent *Pxr* and *Car* genes and the gene clusters *Cyp3a*, *Cyp2c* and *Cyp2d* replaced by counterpart human genes, has recently been reported (*Kapelyukh et al, 2014*).

In the not-to-distant future, microfluidic organs-on-chips may be adopted as a preferred platform for drug testing, replacing animal models. In a microfluidic device, live cells on chips, organized in continuously perfused chambers, mimic the complex multicellular environment so that bioavailability, efficacy and toxicity of test molecules could be assessed in a context which, in part, recapitulates human tissue and organ physiology (*Bhatia & Ingber, 2014; Reardon, 2015*). The future drug discovery

pipeline may also include a workflow that assesses drug-induced PTM profiles of PXR and CAR determined through liquid chromatography-coupled-tandem mass spectrometry, and examines how PTM alters PXR/CAR activity using an approach similar to that reported recently for PXR (*Elias et al, 2014*).

Summary and Perspectives

PXR and CAR, the two nuclear receptors that are activated by drugs and other xenobiotics, coordinate both metabolism of orally administered drugs in the liver and intestine and excretion of drug metabolites by mediating transcriptional induction of genes encoding phase I/phase II drug-metabolizing enzymes (DMEs) and transporters which regulate drug influx (phase 0) and efflux (phase III) of drug metabolites. Phase 0-III mediators are also induced by ligand-activated VDR, especially in the enterocytes of intestine. Additional nuclear receptors, especially FXR, HNF4-α, LRH-1 and SHP regulate expression of the enzymes and transporters involved in cholesterol and bile acid homeostasis. More than 90% of all known drugs are metabolized by a subset of cytochrome P450s (CYPs) -- CYP3A4/3A5, CYP2D6, CYP2B6, CYP2C9, CYP2C19, CYP1A2, CYP2C8, CYP2A6, CYP2J2 and CYP2E1. In the human liver and intestinal epithelium, CYP3A4 and its functionally indistinguishable isoform CYP3A5 are the most abundant CYP enzymes and together, they metabolize more than half of all prescription medicines. Overdosing or underdosing leading to drug toxicity or reduced drug efficacy, respectively, is the consequence of interference from a co-administered second drug (DDI, i.e. drug-drug interaction) or from a dietary or herbal agent (drug-food/drug-herb interaction). Adverse (or beneficial) drug interaction results from i) enhanced gene transactivation for DMEs or transporters due to PXR/CAR activation by the interfering drug or other agent; and/or ii) altered DME or transporter activity. In order to minimize late-stage failure of clinical trials, an essential routine at early stages of drug development is to evaluate candidate molecules for effects on the activities and expression of a select set of CYP isozymes; for PXR and CAR activation and for drug-drug interaction (DDI).

Humanized mouse strains where murine *Cyp*, *Pxr* and *Car* genes are exchanged for human counterparts, as in hPXR-hCAR-hCYP3A4-hCYP3A7 mice (available commercially from Taconic, NY) or recently reported hPXR-hCAR-hCYP3A4/3A7-hCYP2C9-hCYP2D6 mice, may replace a cell-based workflow for screening candidate drugs. A humanized mouse model provides human-like drug metabolism machinery and *in vivo* relevance. A microfluidic organ-on-a chip platform, which mimics human physiology at tissue and organ levels, may be used in the near future as a preferred alternative to animal models for screening drug candidates (**Fig. 5**).

Disparate drug response among individuals results from altered activity or expression of DMEs/ transporters due to single nucleotide polymorphisms (SNPs) in coding regions or in PXR-/CAR-/VDR/HNF4-α-regulated genomic loci; it can also be due to SNPs of PXR/CAR/VDR/HNF4-α that lead to variable expression or activity of these nuclear receptors. An epigenome signature is specified by DNA methylation, chromatin histone marks for transcription activation/repression (largely defined by lysine acetylation and lysine/arginine methylation of the amino-terminal tails of H3 and H4 histones), and by non-coding regulatory RNAs (microRNAs, long ncRNAs). The signature can have a profound impact on drug metabolism and disposition due to changes in PXR/CAR/VDR mediated transactivation of phase 0-III genes. The epigenome landscape also contributes to interindividual variations in drug response, since such a landscape is shaped by both endogenous regulatory molecules and exogenous factors that are as varied as lifestyle, food habits, pollution and psychological disposition.

An integrated scheme linking genetic and epigenetic factors to drug metabolism/disposition, and interindividual variations in drug response is presented (**Fig. 6**). In the era of personalized medicine, all of these regulatory factors must be taken into consideration before deciding on a medicinal regimen that provides optimal therapeutic efficacy and minimal toxicity, while preventing adverse drug reactions.

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FIGURE LEGENDS

Figure 1. **Steps involved in drug metabolism and disposition.** (1) Uptake transporter mediates drug entry into the cell. (2) Drug activates xeno-sensing NR in the cytoplasm or nucleus. (3) NR binds to XREs in target genes that are involved in drug metabolism and clearance. (4) Coactivator association with the DNA-bound NR and a cascade of activating steps, which culminate in gene transcription for DMEs, transporters. (5) Expression of phase 0-III mediators. (6) Phase I enzyme adds water-soluble functional groups to the drug structure. (7) **A** phase II conjugative transferase adds hydrophilic groups to

drug/drug metabolite. (8) Phase III efflux transporter moves to plasma membrane. (9) Transporter-assisted drug efflux. (10) Drug clearance through biliary and urinary excretion.

Figure 2: **Percentage of all prescription drugs metabolized in human liver by a particular CYP enzyme**. (adapted from *Zanger & Schwab, 2013*).

Figure 3. **Induction of the human *SULT2A1* promoter by PXR, CAR and a synergizing effect of HNF4-α.** Schema showing a PXR- and CAR-binding composite XRE comprised of IR2 and DR4 elements, and an HNF4-α-binding DR1 element located downstream of XRE. Dotted, upward arrows signify promoter induction. Interplay of DR1-bound HNF4-α with XRE-bound PXR/RXR, CAR/RXR results in a synergistic effect (triple upward arrows) on SULT2A1 induction. (the diagram is based on results described in *Echchgadda et al, 2007*).

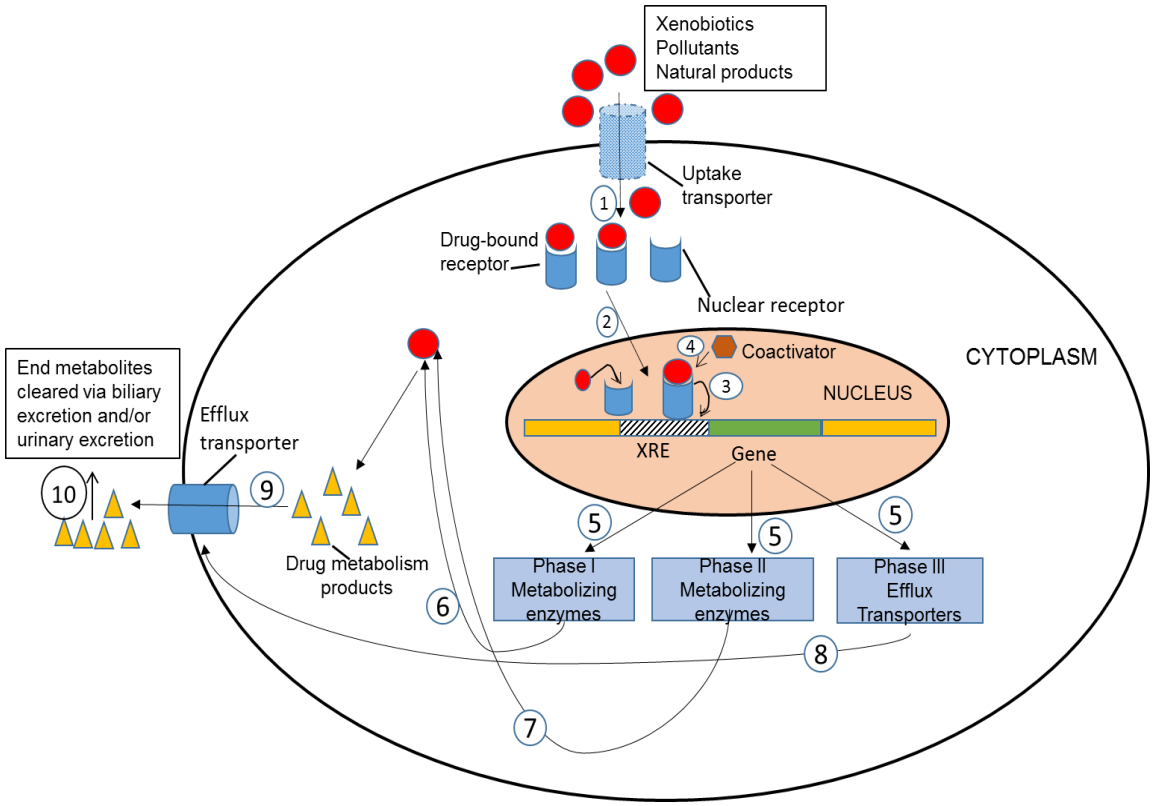
Figure 4. **SULT2A1 mRNA induction by cholic acid in mouse liver.** Sult2A1 mRNAs in mouse livers were assayed by semi-quantitative RT-PCR. Cholic acid, a primary bile acid, was added to diet at 1% w/w. Data are for 3 individual mice (6-month-old, male) from the control and experimental group. Levels of β-actin mRNAs served as the normalization control (*B. Chatterjee & CS Song, unpublished*)

Figure 5. **Overview of drug-screening platforms**: Candidate drugs are screened for effects on the activity and expression of a select set of CYPs (e.g. CYP3A4, and several other CYPs). Workflow for traditional screening (shown at left) relies on cell-based high throughput assay to identify and narrow down candidates with potential for optimal drug activity. Microfluidic organ-on-a chip constitutes an emerging technology that may replace cell-based screening as the primary assay platform. In cross screening, cells are co-administered with a test drug and a second drug or a non-drug xenobiotic agent

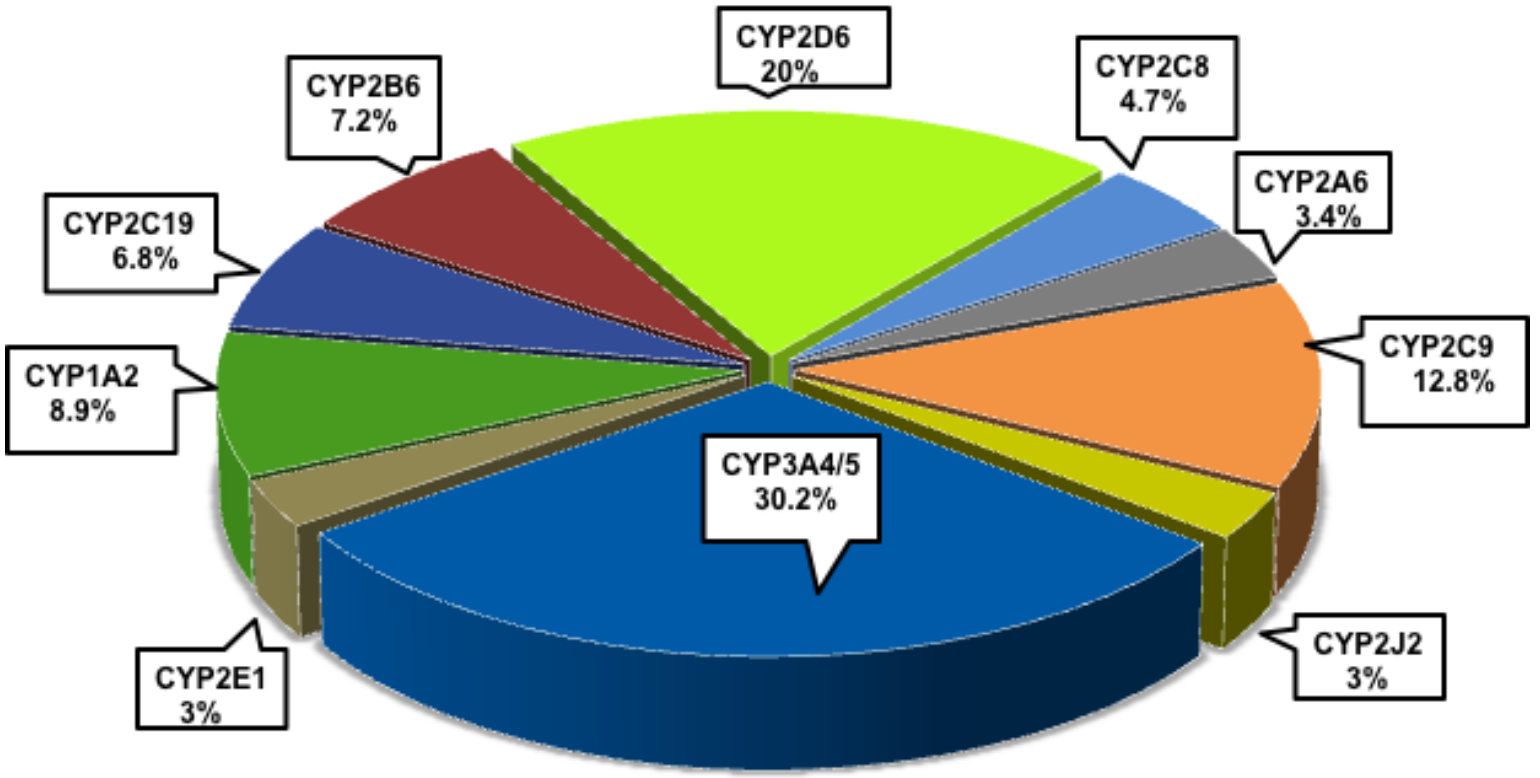
(such as a medicinal herb or a foodstuff) in order to reveal drug-drug or drug-herb or drug-food interactions. Subsequently, drugs are tested in mice. A humanized mouse model (transgenic mice with human PXR, CAR and CYP genes replacing the counterpart rodent genes) can serve as a human surrogate for the examination of drug interactions in the preclinical stage of drug screening.

Figure 6. **NR-mediated regulation of drug metabolism, drug disposition: control at multiple steps.** Transcriptional regulation primarily dictates NR expression and its cellular abundance (box at the upper right corner). Post-translational modification modulates NR stability and NR activity (box at upper left corner). (A) Drugs activate xenobiotic NRs (PXR, CAR), which in turn modulate the expression of phase 0-III mediators via induction of XREs. Ligand-activated VDR also induces DME and transporter expression. (B) Drug-drug, drug-herb, drug-food interactions cause altered NR expression/activity leading to altered expression of DME/transporter. An interfering agent (such as a second drug or a dietary constituent) may also modulate DME/transporter activity via competitive or allosteric regulation. (C) Histone modification and DNA methylation modulate NR expression; they also modulate NR-regulated DME/transporter expression due to modified histones/DNA methylation at or near XREs. (D) SNP at an XRE or at an alternate regulatory locus of phase 0-III genes leads to a change in NR interaction with the response element, which alters DME and transporter expression. SNP in coding regions of PXR/CAR/VDR/HNF4-α and DMEs/transporters is known to alter the activity or cellular abundance of these proteins/enzymes. (E) Micro RNAs, long non-coding RNAs (lncRNAs) regulate the cellular abundance of NRs and mediators of phase 0-III processes. (F) Interindividual differences in drug response result from SNP at an XRE or at another NR-interacting regulatory locus of the target gene. SNP has also been detected at coding regions of NRs or phase 0-III mediators.

Prakash et al, Fig. 1



Prakash et al, Fig. 2



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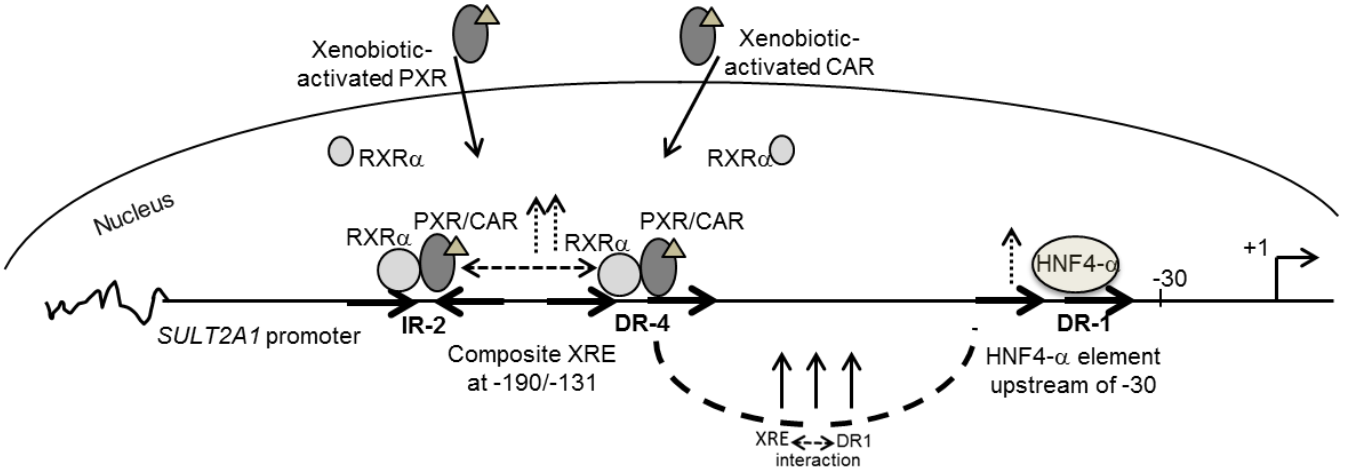

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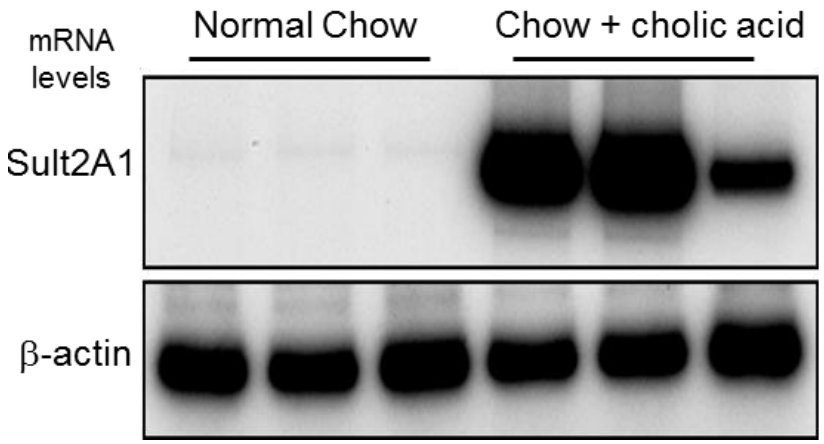
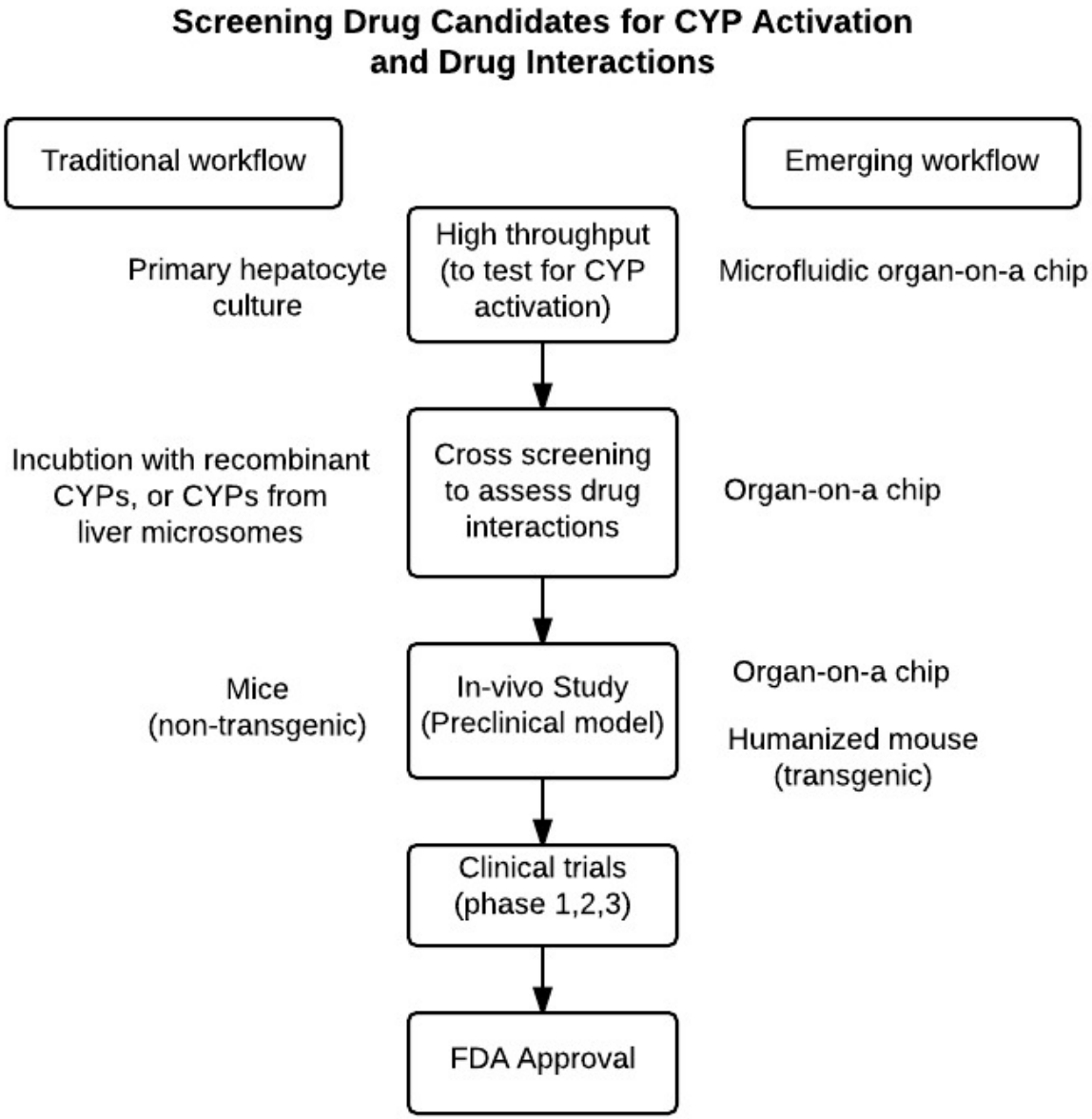


Prakash et al, Fig. 5



Prakash et al, Fig. 6